REMARKS

I. Explanation of Amendments to the Specification

The amendments at pages 7, 14 and 16 correct obvious typographical errors. The paragraph beginning on page 7 was amended to correct the erroneous reference to the "peptides of SEQ ID NO: 3." As Figure 2 and the sequence listing demonstrate, SEQ ID NO: 3 is a nucleic acid sequence. In the foregoing amendment, the reference to peptides at page 8, lines 16 and 18, now refers to the "peptides of SEQ ID NO: 4" (SEQ ID NO: 4 is encoded by SEQ ID NO: 3). In addition, the amendment omits references to claims 14, 15 and 16 at page 14. The reference of "Swiss Mutation" at page 16 was a typographical error as evidenced by the references to "Swedish mutation" throughout the specification, for example at page 13, line 6-7, page 22, lines 15-17, page 41, lines 4-5 and page 74, line 7. These amendments do not add new matter to the specification.

In addition, the specification at page 32 describes tyrosine as a hydroxyl residue similar to serine. The correct statement should refer to threonine as a hydroxyl residue and this misstatement is corrected by the foregoing amendment. This was an obvious typographical error as tyrosine is described in the specification, at page 32, line 33 through page 33, line 1, as an aromatic residue. This amendment does not add new matter to the specification.

II. The Rejection Under 35 U.S.C. § 102(e) Should be Withdrawn

The Examiner rejected claims 1, 3 and 4 under 35 U.S.C. § 102(e), alleging that the claims are anticipated by U.S. Patent No. 6,319,689 (denoted herein as Powell *et al.*), as evidenced by Vassar *et al.* (*Adv. Drug Delivery Rev.* 54(12): 1589-1602, 2002). The Examiner alleged that Powell *et al.* discloses a polynucleotide that is 98.2% identical to SEQ ID NO: 3 and the polynucleotide sequence of Powell *et al.* encodes an aspartyl protease polypeptide that is 99.8% identical to SEQ ID NO: 4. The Examiner also alleged that Powell *et al.* teaches fragments of the polypeptides that retain aspartyl protease activity and variants with conservative substitutions that also retain aspartyl protease activity. In addition, the Examiner stated that Powell *et al.* discloses the complement of the polynucleotide and teaches hybridization of nucleic acid molecules to the polynucleotides and complements thereof. The Examiner also correctly noted that Powell *et al.* does not expressly teach that the aspartyl protease processes APP into amyloid beta, but the Examiner indicated that

Vassar *et al.* teaches that the claimed activity is an inherent property of the aspartyl protease. The Applicants traverse this rejection.

Anticipation requires that a prior art reference disclose a product (in this case polypeptides) that satisfies every limitation of a claim. Powell *et al.* does not teach every limitation of claim 1 or by extension, any of claims 3 or 4, because they depend from claim 1.

Part (a) of claim 1 specifies a polypeptide that comprises an amino acid sequence set forth in SEQ ID NO: 4. The Examiner agrees that the sequence in Powell *et al.* differs from SEQ ID NO: 4 (less than 100% identity), so Powell *et al.* does not anticipate the subject matter of part (a).

Part (b) of claim 1 specifies a polypeptide that comprises fragments of SEQ ID NO: 4 that exhibit aspartyl protease activity involved in processing APP into amyloid beta and include the aspartyl protease active site tripeptides DTG and DSG. Powell *et al.* differs from SEQ ID NO: 4 at position 130, which falls within the domain defined by active site tripeptides DTG and DSG. Thus, Powell *et al.* does not anticipate the subject matter of part (b).

Parts (c) of claim 1 specifies a polypeptide that comprises an amino acid sequence that is a conservative substitution variant of the polypeptides of parts (a) and (b), wherein the only amino acid differences are conservative substitutions otherwise the polypeptide sequence is identical to the sequences of parts (a) and (b). The genera of polypeptide defined by part (c) does not include the sequence taught by Powell *et al.* because the amino acid sequence disclosed in Powell *et al.* has a Glu at position 130, while SEQ ID NO: 4 has a Val at position 130. A substitution of Val for Glu is not a conservative substitution, as Glu is an acidic residue and Val is an aliphatic residue. (Likewise, Powell *et al.,* does not anticipate the polypeptide of claim 19 with aspartyl protease activity, which is identical across its length to a sequence in SEQ ID NO: 4).

Vassar *et al.* is a review article that discusses the clinical significance of BACE1 (also known as β-secretase or Asp2). The amino acid sequence provided in Fig. 2 of Vassar *et al.* teaches the amino acid sequence of SEQ ID NO: 4 of the present invention and not the amino acid sequence taught in Powell *et al.* Accordingly, the amino acid sequence taught in Vassar *et al.* has a Val at position 130. The difference in the Powell *et al.* and

Vassar *et al.* amino acid sequences is within the domain defined by the active site tripeptides. Therefore, Vassar *et al.* does not inherently teach an activity for the Powell *et al.* protein.

Claim 4 specifies that the polypeptide lacks a transmembrane domain. The present application explains that the full length Asp2 protein has a transmembrane domain near its C-terminus. This discovery would not have been expected from other aspartyl proteases that had been described in the art, such as renin, pepsinogen, pepsin, and cathepsin D, which did not reportedly have transmembrane domains.

Powell *et al.* purports to disclose a deduced amino acid sequence of a protein called Asp2, but Powell *et al.* fails to teach or suggest that Powell's Asp2 has a transmembrane domain at all, and certainly does not teach to remove a transmembrane domain for any reason. Likewise, Powell *et al.* does not by chance teach any specific Asp2 fragment that lacks the region identified by the Applicants as a transmembrane domain of Asp2 in the present application. Thus, Powell *et al.* does not anticipate the subject matter of claim 4.

Thus, claims 1, 3 and 4 do not read on subject matter disclosed or suggested by Powell *et al.*, and the rejection for anticipation should be withdrawn.

III. Rejection Under 35 U.S.C. § 112, First Paragraph for Lack of Adequate Written Description Should be Withdrawn

The Examiner rejected claims 1, 3, 4 and 16-19 under 35 U.S.C. § 112, first paragraph, alleging the specification fails to describe the claimed subject matter in such a way to reasonably convey to one of skilled the art that Applicants were in possession of the invention at the time of filing. The Applicants traverse this rejection.

At the outset, Applicants request that independent claims 17-19 be examined on their merits rather than be lumped with claim 1. These claims contain limitations of sequence identity to a reference sequence. As such, the Examiner's concern as to the structure and activity of variants is misplaced.

The main basis for rejection appears to be an assertion that the specification does not adequately describe all of the variants embraced by independent claim 1.

Part (a) of claim 1 specifies that the polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 4. Due to the well known degeneracy of the genetic code,

SEQ ID NO: 4 defines the structure of the entire genus of polynucleotides that encode it, for a person of ordinary skill.

Part (b) of claim 1 recites fragments of (a) that include the aspartyl protease active site tripeptides DTG and DSG and exhibit aspartyl protease activity involved in processing APP into amyloid beta. There are only one DTG and one DSG in SEQ ID NO: 4, so the genus defined by part (b) is also well defined by SEQ ID NO: 4. Fragments that retain aspartyl protease activity are described at page 30, lines 3-21. Aspartyl protease activity involved in processing APP into amyloid beta is described in the specification at page 1, lines 25-27, page 40, lines 25-27, and pages 55-58 (Example 7). Fragments that include the aspartyl protease active site tripeptides DTG and DSG are described in the specification at page 30, lines 9-19. The application also provides aspartyl protease assays and substrates for the enzyme. Thus, the specification adequately describes the subject matter of part (b).

Part (c) of claim 1 recites that the polypeptide is a conservative substitution variant of (a) or (b), wherein the conservative substitution variant is identical to (a) or (b) except for conservative substitutions and is encoded by a nucleic acid molecule that hybridizes under stringent conditions to complement of SEQ ID NO: 3. In addition, the conservative substitution variant exhibits aspartyl protease activity involved in processing APP into amyloid beta. Conservative substitutions are described in the specification at page 32, line 26, through page 33, line 2.

The limitation specifying that the polypeptide is encoded by nucleic acid molecule that hybridizes under the following stringent hybridization conditions to the complement of SEQ ID NO: 3 (i) hybridization at 42°C in a hybridization buffer comprising 6x SSC and 0.1% SDS, and (ii) washing at 65°C in a wash solution comprising 1x SSC and 0.1% SDS reduces the number of variants encompassed by the genus. Example 9 of the Patent Office's Written Description Guidelines Training Materials provides a hypothetical invention involving nucleic acid sequence that hybridize under highly stringent condition to the complement of a disclosed sequence and the nucleic acid encodes a protein with a recited activity. The Patent Office's opinion regarding such a claim is that one of skill in the art would not expect substantial variation among the species encompassed by the claim because the recited hybridization conditions yield structurally similar nucleic acids. Therefore, the disclosed sequences are considered a representative number of species in view of the

structural and functional requirements of the claim and therefore the genus of polypeptides is adequately described. The further limitation that the differences are conservative substitutions notably reduces the species encompassed by the claimed genus of polypeptides of part (c). The Patent Office's analysis of the written description requirement in the hypothetical does not concern itself with whether the specification provided specific guidance about specific changes at specific positions in the sequence because the genus is sufficiently described by the hybridization limitation, the reference sequence and the biological activity. Or stated differently, the combination of limitations used by the Applicants has been recognized by the Patent Office to provide sufficient specificity and limit variability enough to satisfy the written description requirement.

Claims 16-19 are directed to additional variant polypeptides, which are biologically active aspartyl proteases with a valine at a position that corresponds to position 130 of SEQ ID NO: 4. In claim 16, the polypeptide is encoded by a nucleic acid that hybridizes to SEQ ID NO: 3 under stringent wash conditions. According to Example 9 of the Written Description Guidelines described above, claim 16 is adequately described by the specification. In claim 17, the polypeptide is encoded by a nucleic acid which is identical across its length to the sequence set forth in SEQ ID NO: 3. In claim 18, the polypeptide is encoded by nucleic acid which is identical to a sequence set forth as SEQ ID NO: 3, and claim 19 is directed to a polypeptide comprising an amino acid sequence which is identical across its length to a sequence in SEQ ID NO; 4. Claims 17, 18 and 19 are adequately described in the specification for the same reasons that polypeptides of claim 1(a) or 1(b) are adequately described.

The "Summary of Invention" section of the patent application contemplates and describes polypeptides having aspartyl protease activity and characterized by a first tripeptide DTG and a second tripeptide DSG and 100-300 amino acids in between these tripeptides or "special amino acids." (See, *e.g.*, pp. 3-9; see also p. 26, lines 23-27 of the detailed description.) In SEQ ID NO: 4, these tripeptides occur at positions 93-95 and 289-291. References to these signature sequences, which help define the protease active site of Asp2, are incorporated into the claims to clarify that the claimed polypeptide fragments do not encompass short peptides lacking an active site. The minimum active site can be precisely defined using the activity assays taught in the applications, *e.g.* at pages 74-75. The

application also provides significant guidance with regard to signal peptide and propeptide domains of the full length Asp2 polypeptide that are not required for activity. For example, the domains of SEQ ID NO: 4 are defined by amino acid number at page 19, line 24 through page 20, line 3 as follows: the signal sequence spans residues 1-21, the pre-propeptide spans residue 22-45, the propeptide extends to residue 57, the transmembrane domain spans residues 455-477, the cytoplasmic domain spans residues 478-501. The specification identifies a putative alpha helical spacer region that spans residues 420-454 (located between the catalytic domain and the transmembrane domain). (See page 21, line 2-3) This region has cysteine residues that may be implicated in disulfide bonding of the full length or transmembrane deleted Asp2 protein.

In view of the foregoing remarks, claims 1, 3, 4 and 16-19 are adequately described in the specification. Applicants request that the rejection under 35 U.S.C. § 112, first paragraph, for lack of adequate written description be withdrawn.

IV. Rejection Under 35 U.S.C. § 112, First Paragraph for Lack of Enablement Should be Withdrawn

The Examiner rejected claims 1, 3, 4 and 16-19 under 35 U.S.C. § 112, first paragraph alleging that the specification does not enable the full scope of the claims. In particular, the Examiner stated that the specification does not reasonably provide enablement for polypeptide variants. Applicants traverse this rejection.

As with the written description rejection, the Applicants request independent evaluation of claims 17-19 because the sequence identity limitation of these claims render moot many of the issues raised with respect to "variants."

The specification provides a method for identifying variant polypeptide and polypeptide fragments of SEQ ID NO: 4 (see Examples 1 and 2 of the specification; pages 41-47). In particular, the specification teaches how to screen databases for nucleic acid sequences encoding polypeptides comprising the hallmark aspartyl protease active site tripeptides, and to screen human cDNA libraries once the sequences are identified. The polynucleotide can then be cloned from natural sources. Once the nucleic acid sequences of the invention are identified, the specification teaches how to recombinantly express the polypeptides of the invention using expression vectors and host cells (see page 34, line 25 through page 38, line 4 through page 39, line 29). In addition, the specification teaches how

to recover and purify polypeptides from tissues, cultured cells or recombinant cell cultures (see page 34, lines 8-24). The specification also teaches methods for obtaining polynucleotide variants by mutating native nucleotide sequences, such as oligonucleotide-directed mutagenesis (see page 32, lines 3-25).

In addition, the specification teaches assays to determine if the polypeptide retains aspartyl protease activity involved in processing APP into amyloid beta (Example 12, pages 74-75). The amino acid sequences that serve as substrates for an enzyme involved in APP processing are taught in the application and are known in the art. (See Haass *et al.* Cell 75: 1039-1042, 1993; Exhibit A and Citron *et al., Neuron* 14: 661-70, 1995, Exhibit B). The specification teaches that active fragments and variants of the Asp2 aspartyl proteases include the aspartyl protease active site tripeptides DTG and DSG; and these tripeptides are necessary for the fragment to retain activity (page 30, lines 9-14 of the specification).

The specification adequately enables polypeptide fragments and conservative substitution variants of the amino acid sequence of SEQ ID NO: 4. The Applicants disclosed two amino acid sequences (SEQ ID NOS: 6 and 8) which are at least 95% identical to the amino acid sequence of SEQ ID NO: 4. Example 12 demonstrates that, in addition to having 95% identity to SEQ ID NO: 4, the Asp2 polypeptide of SEQ ID NO: 6 possesses APP processing activity similar to Asp2 polypeptide of SEQ ID NO: 4. The Applicants also disclosed the amino acid sequence of murine Asp2(a) as SEQ ID NO: 8, which is greater than 95% identical to SEQ ID NO: 4. Further, in Example 3 the Applicants provide a working example which enables one of skill in the art to isolate Asp2 polypeptides which have 95% identity to SEQ ID NO: 4 from a cDNA library. (See page 41, line 1 through page 49, line 4). Polypeptides that encoded by a nucleic acid sequence that is highly similar (*e.g.* 95% identical) to another nucleic acid sequence are expected to hybridize under stringent conditions.

In addition, the specification adequately enables polynucleotides that hybridize to the complement of SEQ ID NO: 3 under the recited stringent hybridization conditions. Methods of detecting polynucleotides that hybridize to a particular nucleotide sequence are well known in the art. The claims require that the polynucleotide hybridize under stringent conditions, and under these conditions one of skill in the art would not expect

substantial variation among the species encompassed with the scope of the claim (see page 36 of the U.S. Patent and Trademark Office Revised Written Description Guidelines).

The claims specifically define the differences in the variant polypeptide sequence as conservative substitutions. Groups of amino acids with similar physiochemical properties are well known in the art (See Stryer pages 18-21, attached hereto as Exhibit C). The term is also recognized by the U.S. PTO and is common in the art (See MPEP § 2144.08). This claim limitation significantly reduces the number of variant polypeptides encompassed by the structural limitation of the claims, and increases the percentage that would be shown, through routine screening, to retain enzymatic activity (the functional limitation of the claims). Contrary to the Examiner's basis for rejection, the specification, in view of the recited claim limitations, provides adequate guidance as to the common features of the claimed genus and guidance as to which regions of the polypeptides of the invention may be conserved to maintain aspartyl protease activity.

The Examiner asserted that the specification does not provide the structural requirements of the amino acid sequences encompassed by the claims and it is unpredictable which variations meet the limitations of the claims. The structural components necessary for aspartyl protease activity are taught in the specification, and the claimed fragments are required to have the aspartyl protease active site tripeptides DTG and DSG. The specification, at page 25, lines 25-30, teaches that aspartyl proteases possess a two domain structure which folds to bring two aspartyl protease residues into proximity of the active site and the active site is embedded in the short tripeptide motifs DTG and DSG. Therefore, small or inactive fragments and variants are not encompassed by the claims. The structural and functional limitations in the claim provide the necessary guidance for one of skill in the art to make and use the polynucleotides of the present invention. Moreover, the assays provided in the application allow one to determine whether any particular polypeptide variant is active using only routine screening, and routine screening does not constitute "undue experimentation" under the law.

Furthermore, the specification describes how to make fragments of SEQ ID NO: 4 with the transmembrane domain deleted. In addition, Example 8 (pages 58-63) demonstrates that the transmembrane deleted fragments are active. The specification also teaches that the polypeptide of SEQ ID NO: 4 has a pre-propeptide that spans residues 22-45

and a propeptide that spans residues 46-57. (See page 19, lines 26-30). One of skill in the art understands that these peptide may be deleted to create an active fragment. The specification also teaches assays for measuring the aspartyl protease activity of the fragments. For example, a novel cell line for measuring processing of APP into amyloid beta is taught at page 40, lines 25-31. These cells can be transfected with a polynucleotide of the invention that expresses the claimed fragment. At pages 51-57, the Applicants teach human cell lines that process APP which provide a means for screening for APP processing activity. Production of amyloid beta peptide in culture can be measured by EIA as described at pages 53-54. Example 12 (pages 74-75), provides cell-free assays using synthetic peptide substrates to measure the aspartyl protease activity of the fragments.

The Patent Office cites the Federal Circuit's *Wands* decision and cites the "*Wands* factors," and the Applicants agree that these principles govern an enablement analysis. However, these principles have been misapplied.

Wands involved screening of large numbers of hybridomas to identify specific hybridomas that fell within the claim limitations. Because the patentee in Wands provided sufficient guidance to make and screen the hybridomas and presented working examples, that the enablement requirement was fulfilled. In re Wands, 858 F.2d 731, 740 (Fed. Cir. 1988). In re Wands does not hold that a specific number of working examples is required. In reaching a decision, the court in Wands considered that the inventor's disclosure provides considerable direction and guidance on how to practice the invention and presents working examples. Id at 740. This fact, coupled with the high level of skill in the biotechnology arts, rendered the invention enabled, according to the Court. Id. Although a considerable amount of work may have been required to do the making and screening, such experimentation is routine, not "undue," according to Federal Circuit's decision in Wands.

In the present application, the claims of the application are directed to a genus of polypeptides with limited variation from a wild-type protease sequence, and required to retain the protease activity. The specification fully discloses methods to make the claimed polypeptides and methods to determine whether these polypeptides exhibit protease activity toward substrates (also taught in the application). Similar to Wands, the invention provides a composition that cleaves (rather than binds) to a specific target, with the target cleavage identified using well-known screening methods. In the many years that have passed since the

invention at issue in Wands, when the level of skill was already considered high, the level of skill in the rapidly advancing arts of DNA and protein manipulation, synthesis, and screening are clearly very high.

In fact, the making and screening required by the present invention (polypeptide identification and enzymatic testing) is much simpler and faster – more routine -- than the making and screening of hybridomas and antibodies set forth in the facts of *In re Wands,* which the Court said was not undue experimentation. Experimentation, even if extensive, is not necessarily undue if it is routine in the art (*In re Wands*, 858 F.2d 731 (Fed. Cir. 1988)).

Notwithstanding these favorable considerations relative to *Wands*, and the fact that polypeptide identification, production and screening is much more routine than *Wand's* hybridoma synthesis, monoclonal production, and monoclonal screening, the Examiner for this case reaches the opposite conclusion from the conclusion drawn by the Federal Circuit in *Wands*. Notwithstanding the lip-service paid to *Wands*, the Examiner refuses to recognize that the application teaches methods for making polypeptides of varying sequence using techniques common in the art such as recombinant expression, and teaches assays, many *in vitro*, by which the polypeptides and fragments can be routinely screened.

In addition, the Examiner cited to Mickle *et al.* (*Med. Clin. North Amer.* 84(3): 597-607, 2000), Voet *et al.* (*Biochemistry,* 1990 John Wiley & Sons, pgs. 126-129 and 228-234) and Yan *et al.* (*Science* 290: 523-527, 2000) to demonstrate that a single amino acid change can alter the function of a protein. Mickle *et al.* describes point mutations that are thought to be involved in the pathogenesis of cystic fibrosis. Voet *et al.* teaches that a single amino acid substitution thought to be involved in the pathogenesis of sickle cell anemia. Yan *et al.* teaches that alteration of two amino acids in a protein can change the receptor to which the protein binds. Each of these reports are isolated examples, and none of these reports were specifically directed to the question of what percentage of mutations in their respective proteins are silent versus activity-destroying. The authors and editors presumably had little interest in publishing about silent mutations. The identification of point mutations that have pathogenic implications is of great interest to the medical and research community and are published, but the studies have no probative value for assessing the likelihood of success at generating active variants. As described above, the present

specification provides nucleic acid sequences encoding the human and murine Asp2 amino acid sequences and therefore one of skill in the art can determine the conserved residues using simple computer alignment programs, which are referred to within the specification. In addition, the cited articles are not relevant because the present specification teaches the protease activity of the encoded polypeptides and the claims are directed to polypeptides with minor sequence variations that retain protease activity. The function of the variant polypeptides may be determined by routine screening for cleavage of APP as described in the specification.

For the foregoing reasons, the specification provides reasonable enablement of claims 1, 3, 4 and 16-18. Therefore, Applicants request that the rejection under 35 U.S.C. § 112, first paragraph for lack of enablement be withdrawn.

V. Declaration of Michael Bienkowski, Ph.D.

Submitted herewith (as Exhibit D) is a Declaration of Michael Bienkowski, Ph.D., that was submitted during prosecution of related U.S. patent applications.

VI. Obviousness-Type Double Patenting Rejections

Claims 1, 3, 4 and 16-19 were rejected under the judicially created doctrine of obviousness-type double patenting in view of the following patents: U.S. Patent Nos. 6,913,918, 6,825,023 and 6,828,117. In addition, claims 1, 3, 4 and 16-18 were provisionally rejected under the judicially created doctrine of obviousness-type double patenting in view of the co-pending patent application no. 10/940,867.

Applicants request that these double patenting rejections be held in abeyance until there is an indication of allowable subject matter. At that time, Applicants will consider filing appropriate disclaimer(s). It is premature to disclaim term before the scope of an allowable claim is clear.

VII. Related Applications

On page 7 of the Office Action, the Examiner requested that the Applicants provide an updated listing of the related patent applications such as the listing provided with the Information Disclosure Statement dated April 5, 2005. Submitted herewith, as Appendix E, is and updated list of issued U.S. patents and pending U.S. patent applications that are related to the above-identified application. The related applications claim priority to U.S.

provisional applications 60/101,594 and 60/155,493, U.S. application 09/404,133 (abandoned) and U.S. application 09/668,314. One or more of the related applications may contain claims that are similar in scope or content to claims of the present application. Copies of these applications are not enclosed, but are pending in the U.S. Patent Office and should be available to the Examiner.

During the course of prosecution of these applications, different examiners have raised a variety of rejections under 35 U.S.C. §102, §103, §112, first and second paragraphs, and double patenting. Upon request, the Applicants will provide the Examiner with copies of office actions and/or responses filed for the related applications. The Examiner is invited to contact the undersigned if further explanation of the patent family is necessary.

CONCLUSION

In view of the foregoing remarks, Applicants believe claims 1, 3, 4 and 16-18 are in condition for allowance and early notice thereof is solicited.

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Cellular Processing of β-Amyloid Precursor Protein and the Genesis of Amyloid β-Peptide

Minireview

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In 1907, Alois Alzheimer described a novel brain disease that principally affects late middle-aged and older humans and results in a progressive and ultimately fatal loss of mental capacity, particularly recent memory. This disorder, later called Alzheimer's disease (AD), is defined by characteristic neuropathological lesions in brain regions important for intellectual function. The only invariant pathological change is the formation of extracellular amyloid plaques in the cerebral and limbic cortices and chemically similar amyloid deposits in the walls of meningeal and cerebral blood vessels. Other structural changes found in AD brain, including the intraneuronal neurofibrillary tangles, are not restricted to AD but also occur in numerous etiologically diverse neurodegenerative diseases, and they are sometimes sparse in AD itself.

The Amyloid β-Peptide Plays an Early Role in the Pathogenesis of AD

The major proteinaceous component of the vascular and plaque amyloid deposits, the hydrophobic 39–43 residue amyloid β-peptide (Aβ), is proteolytically derived from an integral membrane protein, the β-amyloid precursor protein (βAPP; Figure 1) that is encoded by a gene on human chromosome 21 (Kang et al., 1987). Importantly, patients with trisomy 21 (Down's syndrome) develop neuropathological changes indistinguishable from those of AD, but starting at a very early age. The finding that an extra copy of the βAPP gene invariably leads to typical AD pathology that begins with amyloid plaque formation supports the hypothesis that the metabolism of βAPP into Aβ may play a crucial role in the pathogenesis of the disease. Furthermore, synthetic Aβs have been shown to produce toxic effects on cultured neurons (Yankner et al., 1990), although the cellular and molecular mechanisms of Aβ-associated neurotoxicity remain controversial. Perhaps the strongest evidence for a pathogenic role of βAPP emerges from the discovery that some cases of autosomal dominant AD are strongly linked to missense mutations in the βAPP gene, specifically within and immediately flanking the Aβ sequence (summarized by Hardy, 1992). For these reasons, this review will focus on the complex cellular processing of βAPP and particularly on the recent discovery that Aβ is generated continuously by a physiological mechanism.

Conventional Secretory Processing of βAPP Prevents the Generation of Aβ

The primary structure of βAPP closely resembles a cell-surface receptor (Figure 1) with a signal sequence, a large extramembranous N-terminal region, a single transmembrane domain, and a small cytoplasmic C-terminal tail (Kang et al., 1987). Aβ represents only a small fragment of βAPP, and proteolytic processing of the precursor results in the formation of this peptide. The last 11–15 amino acids of Aβ are located within the transmembrane domain (Figure 1), presumably protecting it from proteolytic cleavage. Moreover, Aβ generation is complicated by the fact that normal secretory processing of βAPP (Weidemann et al., 1989) results in a cleavage of the precursor at amino acid 16 within Aβ (Esch et al., 1990). This scission, made by an unidentified enzyme designated α-secretase, leads to the secretion of the large soluble ectodomain of βAPP (APP_s) and the retention of the small 10 kd C-terminal fragment within the membrane (Figure 2A). The α-secretase-mediated cleavage of βAPP can apparently occur at the cell surface (Sisodia, 1992; Haass et al., 1992a) or intracellularly (Sambamurti et al., 1992). Thus, conventional secretory processing of βAPP precludes the formation of Aβ. Based on these data, it has been widely assumed that only aberrant processing under pathological conditions could lead to the formation of Aβ and its release from the cell membrane.

Endosomal-Lysosomal Processing of βAPP Leads to Potential Aβ Precursors

During the last year, evidence has accumulated that only a minority of βAPP molecules are actually processed by this secretory pathway. C-terminal fragments of βAPP were identified in cultured cells (Golde et al., 1992; Haass et al., 1992a) or brain tissue (Estus et al., 1992) that contained the complete Aβ sequence and could thus serve as potential degradative intermediates for Aβ formation. These fragments were found to be stabilized by leupeptin,

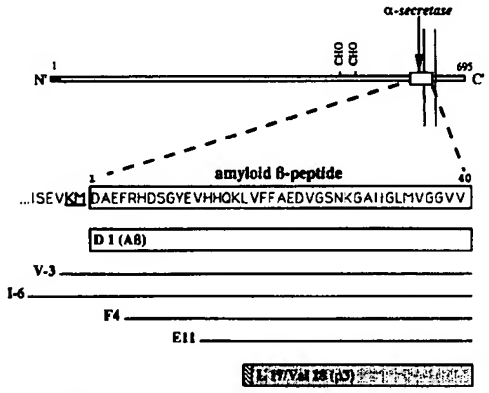

Figure 1. The Primary Structure of βAPP

The vertical lines represent the single transmembrane domain, and the open box represents the Aβ peptide. The lysine and methionine residues that are underlined are substituted by asparagine and leucine, respectively, in the case of the double missense mutation found in a Swedish family with autosomal dominant AD. The horizontal boxes and lines below the Aβ sequence represent peptides that have been found to be secreted by a variety of tissue culture cells. The major species begins at Asp-1, whereas minor peptides begin at Val-(-3), Ile-(-6), Phe-4, and Glu-11. The stippled box corresponds to p3, which starts at Leu-17 (hatched segment) or at Val-18. The C-terminus of secreted Aβ also shows heterogenity, with a major species ending at residue 40.

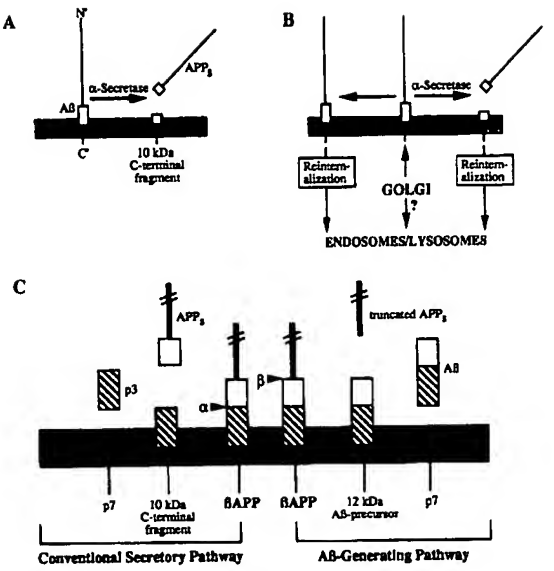

Figure 2. Known Cellular Processing Pathways of βAPP

(A) Conventional secretory processing of βAPP. (B) Endosomal–lysosomal processing of βAPP. (C) Proteolytic generation of Aβ and p3. Arrowheads designated α and β indicate the sites of cleavage of βAPP utilized by unidentified proteases designated α-secretase and β-secretase, respectively. The arrowhead within the transmembrane region of βAPP indicates the sites of the Aβ C-terminal cleavage, again effected by an unknown protease.

ammonium chloride, or chloroquine, agents known to inhibit endosomal–lysosomal proteases. Such results led to the proposal that βAPP could also be processed in an endosomal–lysosomal pathway. However, it is not clear whether some of these fragments may initially be made within the Golgi or at the cell surface and then accumulate within the lysosome, where they could be subjected to further processing and degradation. Surface biotinylation and antibody binding experiments on living cells revealed that full-length βAPP can be reinternalized from the cell surface in an apparent coated pit-mediated pathway (Haass et al., 1992a). Indeed, full-length βAPP and the 10 kd C-terminal fragment have been found within isolated clathrin-coated vesicles (Nordstedt et al., 1993). Furthermore, isolation of late endosomes–lysosomes from leupeptin-treated cells directly demonstrated that full-length βAPP, the 10 kd C-terminal fragment, and a range of slightly larger C-terminal fragments of βAPP containing the intact Aβ sequence accumulate within lysosomes (Haass et al., 1992a). Taken together, these observations indicate that some full-length βAPP molecules are reinternalized from the cell surface and targeted, together with the 10 kd and probably other C-terminal fragments, to lysosomes for final degradation (Figure 2B). In addition to this reinternalization pathway, a pathway that targets βAPP from the trans-Golgi network directly to endosomes and lysosomes could exist.

Normal Cellular Processing of βAPP Leads to the Secretion of Aβ

The description of a normal processing pathway for βAPP that generates Aβ-bearing fragments under physiological conditions suggested that aberrant processing of βAPP might not be necessary to generate Aβ. This concept has now been validated by the unexpected discovery that Aβ is normally secreted into the media of a wide array of cultured cells that express βAPP (Haass et al., 1992b; Seubert et al., 1992; Shoji et al., 1992; Busciglio et al., 1993). In such conditioned media, peptides of 4 kd (Aβ) and 3 kd (p3) were identified by immunoprecipitation with antibodies to Aβ (Figure 2C) (Haass et al., 1992b; Shoji et al., 1992; Busciglio et al., 1993). Radiosequencing indicates that βAPP proteolysis normally yields a heterogeneous population of Aβ and closely related peptides (Figure 1). Within the 4 kd range, the major peptide appears to be identical to Aβ starting at Asp-1 (Haass et al., 1992b; Seubert et al., 1992). Additional peptides have been identified in minor amounts that start at Val-(-3), Ile-(-6), Glu-11, or Phe-4 (Haass et al., 1992b) (Figure 1). In addition to this N-terminal heterogeneity, the C-termini of Aβ in culture medium also vary considerably (Dovey et al., 1993). In the 3 kd range, the peptide begins at or immediately adjacent to the conventional α-secretase cleavage site (Figure 1), suggesting that this fragment arises from the 10 kd C-terminal peptide (Haass et al., 1992b) (Figure 2C, left). These data suggest that the C-terminal cleavages creating the Aβ and p3 peptides are mediated by similar, if not identical, proteases. This C-terminal Aβ cleavage should create an ~7 kd βAPP C-terminal fragment (p7 in Figure 2C). Indeed, small amounts of such a fragment can be detected in lysates of cells transfected with a βAPP cDNA (C. H. and D. J. S., unpublished data). The N-terminal cleavage generating p3 is thus probably made by α-secretase, whereas the N-terminal cleavage generating Aβ appears to be mediated by a highly sequence-dependent protease (Citron et al., 1992; M. Citron and D. J. S., unpublished data) distinct from α-secretase, which has been shown to cleave βAPP in a sequence-independent manner (Sisodia, 1992). This β-secretase cleavage should result in a truncated species of APP$_s$; indeed, such a form of APP$_s$ terminating just before the Aβ region has recently been detected in the media of cultured cells (Seubert et al., 1993) (Figure 2C, right).

Importantly, Aβ has been detected not only in the supernatants of cultured cells but also in normal body fluids, e.g., in human cerebrospinal fluid (Seubert et al., 1992; Shoji et al., 1992). An Aβ-immunoreactive species has been detected in human serum (Seubert et al., 1992), although its chemical identity has not yet been established. These findings indicate that in vitro production of Aβ reflects βAPP processing in vivo.

The observation that Aβ is normally produced by cultured cells provides a dynamic model system in which one can examine the detailed molecular mechanisms leading to Aβ. Heretofore, Aβ had only been obtained, laboriously and in small quantities, from insoluble amyloid deposits of postmortem human brain. As noted above, several missense mutations in the βAPP gene have been identified to date in patients with early-onset familial AD. A key step toward understanding the pathogenesis of AD will be to clarify the effect of these mutations on βAPP processing. So far, no obvious changes in the conventional secretory

processing of these mutant βAPP molecules in vitro have been documented. However, at least one of these mutations, one that was identified in a Swedish family (Mullan et al., 1992) (Figure 1), results in a striking 5- to 8-fold increase in secretion of Aβ in vitro (Citron et al., 1992; Cai et al., 1993). This increase in Aβ production is due to a methionine to leucine switch at the N-terminal cleavage site of Aβ, a substitution that apparently increases the affinity of the substrate for the β-secretase. These findings provide a clear link between a familial AD genotype and the generation of the AD neuropathological phenotype. However, another missense mutation in the βAPP gene at codon 717 (4–6 residues beyond the usual C-terminus of Aβ) has so far not been shown to result in an obvious increase of Aβ production (Cai et al., 1993). Whereas this mutation and others at the same codon (Hardy, 1992) might not affect the quantity of Aβ, they could potentially result in longer, more readily aggregating peptides. It is known that additional amino acids at its C-terminus may enhance the aggregation of Aβ in vitro (Jarrett and Lansbury, 1993).

In additon to the several known missense mutations in βAPP, genes linked to the familial AD phenotype have been localized to chromosome 14 (e.g., Schellenberg et al., 1992) and chromosome 19 (Strittmatter et al., 1993). The responsible gene on chromosome 14 has not yet been identified, but one might speculate that it could be involved in regulating βAPP expression or processing or the metabolism of Aβ itself, since the families linked to chromosome 14 have a very early and severe β-amyloidotic phenotype. On chromosome 19, the gene encoding apolipoprotein E shows segregation of the ε4 allele with late-onset familial AD (Strittmatter et al., 1993). How the normally occurring ε4 polymorphism, which results in a single amino acid substitution in apolipoprotein E, predisposes subjects to AD remains to be determined. One hypothesis suggests that apolipoprotein E might bind to and serve as a carrier of Aβ in vivo. These findings and others clearly indicate that familial AD is genetically heterogeneous. It appears that mutations or polymorphisms in a variety of gene products will ultimately be shown to lead to progressive cerebral β-amyloidosis and AD.

Mechanism of Aβ Production and Regulation

Little is currently known about the cellular mechanisms that allow Aβ generation. Interestingly, Aβ has thus far not been detected intracellularly (Haass et al., 1992b, 1993; Shoji et al., 1992). The complete inhibition of Aβ generation by brefeldin A suggests that transport of βAPP through the Golgi is a prerequisite for Aβ production (Haass et al., 1993). Aβ formation is not inhibited by leupeptin (Shoji et al., 1992; Haass et al., 1993; Busciglio et al., 1993), and Aβ has not been found in isolated lysosomes (Haass et al., 1993). However, agents that interfere with pH gradients in vesicular compartments (NH_4Cl, chloroquine, monensin) markedly inhibit Aβ production in most cell types studied (Shoji et al., 1992; Haass et al., 1993), suggesting that an acidic compartment is necessary for Aβ generation. It is therefore hypothesized that Aβ might be generated in late Golgi vesicles or in early endosomes. With regard to the latter, it is interesting to note that a truncated βAPP construct that lacks the cytoplasmic tail still allows the production of 4 kd peptides, although the large majority of these peptides do not start at Asp-1 of Aβ (Haass et al., 1993). This result suggests that the cytoplasmic domain of βAPP (which contains the NPXY consensus sequence for clathrin-mediated endocytosis) may play a role in targeting the precursor to the subcellular compartment involved in Aβ generation and that reinternalization from the cell surface may favor formation of the peptide.

Whereas all βAPP-expressing cells examined to date process some precursor molecules into Aβ, recent data show that the metabolism of the precursor appears to be a highly regulated process that can be influenced by extracellular signals and intracellular second messengers. These results could potentially be important for the treatment of AD, because they may offer one pharmacological approach to partially inhibiting the production of Aβ. The activation of protein kinase C (PKC) by phorbol esters or certain first messengers can increase the secretion of APP_s (e.g., Nitsch et al., 1992). However, site-directed mutagenesis of potential phosphate acceptor residues in the cytoplasmic domain demonstates that this PKC-mediated effect does not involve enhanced phosphorylation of the βAPP molecule itself; indeed, βAAP is basally phosphorylated solely on its ectodomain (A. Hung and D. J. S., in press). Thus, a distinct protein activated by PKC-mediated phosphorylation is likely to be implicated in βAPP secretory processing, for example, α-secretase itself or a protein involved in the trafficking of vesicles that contain βAPP. Coincident with the enhancement of secretion of APP_s, PKC activation consistently and substantially decreases Aβ formation. This down-regulation is observed after stimulation of the phospholipase C–PKC-linked muscarinic m1 receptor (Hung et al., 1993) or after direct phorbol ester treatment (Hung et al., 1993; Buxbaum et al., 1993).

Based on these data, alternative cellular pathways appear to determine whether βAPP is destined for an amyloidogenic or nonamyloidogenic fate. PKC activation may increase the likelihood that βAPP is cleaved by α-secretase or else it may target βAPP into a cellular compartment that produces lower amounts of Aβ.

Implications for the Potential Therapy or Prevention of AD

The discovery of Aβ generation by cultured cells provides a model system capable of identifying and characterizing pharmacological agents that can down-regulate Aβ production. Furthermore, the ability to quantitate soluble Aβ in biological fluids could lead to a cerebral spinal fluid or plasma assay to monitor Aβ before and during the course of the disease. Chronically lowering Aβ levels in brain, cerebral spinal fluid, serum, or some combination could provide a critical means of slowing the generation of the neuropathological lesions of AD. Decreasing the production of other amyloidogenic precursor proteins has already proven successful in slowing or halting certain potentially lethal amyloid deposition diseases in humans. Further efforts to identify the precise cellular pathway that generates Aβ and to characterize the responsible proteases may provide a particularly attractive route to diminishing the cerebral amyloid burden during aging and in AD.

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Generation of Amyloid β Protein from Its Precursor Is Sequence Specific

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Summary

Cerebral deposition of amyloid β protein (Aβ) is an early and critical feature of Alzheimer's disease. Here we analyze the substrate requirements of proteases ("β-secretases") that cleave the β-amyloid precursor protein (βAPP) at the N-terminus of Aβ (Asp-597 of $βAPP_{695}$) in intact human cells. The cleavage requires a membrane-bound substrate but tolerates shifts in the distance of the hydrolyzed bond from the membrane. The major protease has a minimum recognition region of Val-594 to Ala-598; most substitutions in this sequence strongly decrease or eliminate Aβ production. Only the Swedish familial Alzheimer's disease mutation (K595N/M596L) strongly increases Aβ production. Moreover, in this mutant but not in the wild type, the entire cytoplasmic tail with its reinternalization signals can be deleted without affecting Aβ N-terminal cleavage, consistent with the concept that cleavage of this mutant occurs in a different cellular compartment than that of wild-type molecules. Our results have important implications for current intensive approaches to develop assays for and identify enzymes with β-secretase activity.

Introduction

Alzheimer's disease is characterized by the progressive formation in the brain of insoluble amyloid plaques and vascular deposits consisting of the 4 kDa amyloid β peptide (Aβ; Glenner and Wong, 1984; Masters et al., 1985). Aβ is proteolytically derived from a large integral membrane protein, the β-amyloid precursor protein (βAPP). Four Aβ-containing isoforms of βAPP, comprising 695, 714, 751, or 770 amino acids, have been described (Kang et al., 1987; Ponte et al., 1988; Tanzi et al., 1988; Kitaguchi et al., 1988; Golde et al., 1990). The two longer isoforms contain a serine protease inhibitory domain of the Kunitz type. After maturation within the endoplasmic reticulum and Golgi, βAPP undergoes a constitutive "α-secretory" proteolytic cleavage to release the large soluble N-terminal ectodomain (APP_s) and create a 10 kDa C-terminal fragment that remains membrane bound (Figure 1) (Weidemann et al., 1989; Esch et al., 1990; Oltersdorf et al., 1990; Sisodia et al., 1990). In this pathway, βAPP is cleaved within the Aβ domain primarily between residues Lys-16 and Leu-17, precluding formation of intact Aβ (Esch et al., 1990). Recently, it has been shown that alternative cleavage sites within the Aβ region can be used in different cells, suggesting that α-secretase cleavage is likely to involve a diverse set of proteases (Zhong et al., 1994).

The enzyme(s) responsible for this α-secretory cleavage has not yet been isolated, but indirect evidence suggests that the major form of α-secretase is a membrane-bound enzyme that cleaves βAPP on the plasma membrane (Sisodia, 1992). It has been suggested that the principal determinants of cleavage are an α-helical conformation around the cleavage site and the distance of the hydrolyzed peptide bond from the membrane, not the primary structure in this region (Sisodia, 1992). Aβ must be generated by an alternative pathway that involves at least two proteolytic cuts, one at the N-terminus by an enzyme(s) designated β-secretase and one at the C-terminus by γ-secretase (Figure 1). Thus, a potential therapeutic strategy for decreasing Aβ deposition in Alzheimer's disease involves inhibition of specific enzymes performing one or both of these cuts.

Aβ production and release are normal physiological events. The 4 kDa peptides precipitable by a variety of Aβ-specific antibodies are normally present in the media of βAPP-expressing cultured cells and in human and rodent cerebrospinal fluid. Most of these 4 kDa peptides secreted from human kidney 293 cells, human M17 neuroblastoma cells, and human fetal mixed brain cultures indeed start at Asp-1 (Haass et al., 1992b; Shoji et al., 1992; Seubert et al., 1992; Busciglio et al., 1993) and contain approximately 40 amino acids. Interestingly, the amount of Aβ released into conditioned media is markedly increased by a double missense mutation (K595N/M596L; Figure 1) that occurs in a Swedish Alzheimer's disease family (Mullan et al., 1992), as seen both in transfected cell lines (Citron et al., 1992; Cai et al., 1993) and in primary skin fibroblasts from patients carrying the mutation (Citron et al., 1994). In addition to Aβ, a 3 kDa peptide (p3) starting at Leu-17 is constitutively secreted. This peptide appears to be derived by γ-secretase cleavage of the 10 kDa C-terminal fragment of βAPP following secretion of APP_s (Haass et al., 1993). p3 has recently been detected in diffuse plaques in Alzheimer brain tissue (Gowing et al., 1994).

The precise intracellular site(s) of Aβ generation and the proteases involved in its production are poorly understood. However, recent radiosequencing studies have shown that β-secretase cleavage occurs at slightly different positions in different cell types. In 293 cells, approximately 80% of Aβ produced from wild-type βAPP molecules starts at Asp-1, with the rest beginning at Val(−3) and Ile(−6) (Haass et al., 1992b); however, in primary skin fibroblasts, wild-type βAPP is metabolized to the Asp-1 form of Aβ only (Citron et al., 1994), and in Madin-Darby canine kidney cells, primarily to a form starting at Arg-5 (Haass et al., 1994). These results suggest that different cell-specific proteases are capable of generating the N-terminus of Aβ.

In this study, the substrate requirements of the proteases (β-secretases) that liberate the N-terminus of Aβ in

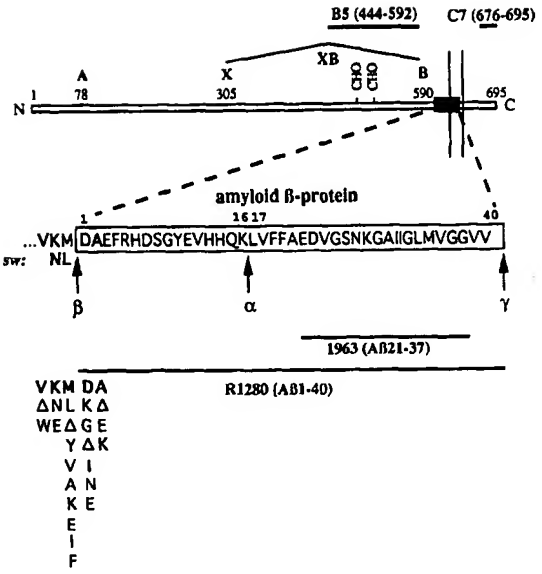

Figure 1. βAPP Structure and Processing

The schematic shows βAPP_{695} with the consensus N-linked glycosylation sites (CHO) and the restriction sites AccI (A), XhoI (X), and BglII (B). The part of the βAPP molecule removed by the XB deletion is indicated. N, N-terminus; C, C-terminus; vertical bars, plasma membrane; black box, Aβ. Solid horizontal lines represent the regions against which antibodies B5, C7, 1963, and R1280 were produced. The amino acid sequence of the Aβ region of βAPP is expanded. The position of the Swedish (sw) FAD mutation is indicated. The sites of the α-, β-, and γ-secretase cleavages are marked by arrows. The exact site(s) of γ-secretase cleavage is unknown, but the majority of Aβ molecules in Alzheimer brain tissue terminate at Val-40 or Ala-42 (Mori et al., 1992; Roher et al., 1993). The amino acids shown in bold type at the lower left were mutated in this study to the amino acids indicated in plain type; Δ signifies a deletion.

293 cells and in SK-N-SH human neuroblastoma cells were analyzed. We show that these proteases depend on a membrane-bound βAPP substrate but tolerate changes in the distance of the cleaved peptide bond from the membrane. We define a minimal recognition sequence, document that the major Asp-1 cleaving enzyme is highly sequence specific, and show that the β-secretase cleavage occurs prior to the γ-secretase cleavage. Further, we show that deletion of the entire cytoplasmic tail of βAPP strongly influences the N-terminal cleavage pattern of wild-type but not of Swedish mutant βAPP, consistent with the idea that the N-terminal cleavage of Aβ in these two βAPP forms occurs in two different intracellular compartments. Finally, we discuss the implications of our results for current intensive efforts to develop an assay for a β-secretase enzyme in human brain and to confirm its identity.

Results

Aβ Production Does Not Require the N-Terminal Portion of βAPP

To define a minimal region of βAPP that is obligatory for Aβ N-terminal cleavage in intact human cells, two βAPP_{695} in-frame deletion constructs, in which the 859 bp fragment from XhoI to BglII (Figure 1) is replaced by a 16 bp synthetic piece of DNA, were analyzed. In these constructs, amino acids 309–589, including the two N-glycosylation sites at 467–469 and 496–498 (Kang et al., 1987), are deleted. Introduction of this deletion into a plasmid carrying the wild-type βAPP_{695} cDNA created plasmid XB, while introduction into a plasmid carrying the βAPP_{695} sequence with the Swedish double missense mutation (Citron et al., 1992) (Figure 1) created plasmid XBsw. Upon transfection, both constructs resulted in the production of Aβ, p3, and APP_s, the latter being about 30 kDa smaller than the respective wild-type protein as a consequence of the deletion (Figure 2A). As with full-length βAPP constructs, the Swedish mutation leads to a strong increase in the amount of Aβ (Figure 2A). This experiment demonstrates that amino acids 309–589 are not specifically required for the β- and γ-secretory cleavages that generate Aβ in intact cells and are not involved in the increase in Aβ production observed in the Swedish mutation. Another internal deletion construct, βAPP_{695}/AX, in which amino acids 78–305, including the ectodomain phosphorylation site(s), were deleted (Hung and Selkoe, 1994) also still allowed Aβ production (Hung and Selkoe, unpublished data), indicating that almost the entire βAPP ectodomain was dispensable with respect to Aβ production.

Proteolytic Generation of the Aβ N-Terminus Tolerates Changes of Distance to the Membrane

It has been postulated that α-secretase cleaves βAPP at a fixed distance from the membrane (Sisodia, 1992). To address whether the same is true for the Aβ N-terminal cleavage, we engineered constructs with amino acid deletions between the Aβ cleavage site and the stretch of Aβ amino acids from 18 to 23, near the transmembrane domain that is required for α-secretase cleavage (Sisodia, 1992). Two deletion constructs were analyzed: in Δ5–9 the amino acids RHDSG of Aβ (601–605 of βAPP_{695}) are deleted, and in Δ9–12 the amino acids GYEV (605–608) are deleted. Both constructs still led to the production of Aβ and p3 peptides, as well as an APP_s molecule that is poorly detectable by the R1280 immunoprecipitating antibody, owing to the deletion (Figure 2B). This finding demonstrates that amino acids 5–12 of Aβ are dispensable for protease recognition of the N-terminal cleavage site. Together with the results of the XB deletion (Figure 2A), it appears that the recognition sequence for β-secretase is limited to a maximum of 11 amino acids (positions 590–600) immediately surrounding the cleavage site. However, we cannot exclude the possibility that residues C-terminal to amino acid 608 might also assist in proper recognition. An analogous insertion construct in which the amino acids IGFEV were inserted after the 7th amino acid of Aβ (position 603) led to a strong decrease in both Aβ and p3, suggesting that this insertion had disrupted the overall structure of the molecule and thereby inhibited both α- and β-secretase cleavages (data not shown). Relative to wild-type βAPP, the Aβ bands derived from both deletion constructs displayed decreased mobility (Figure 2B). This result suggests that the predominant Met–Asp cleavage site was maintained, independently of its membrane distance.

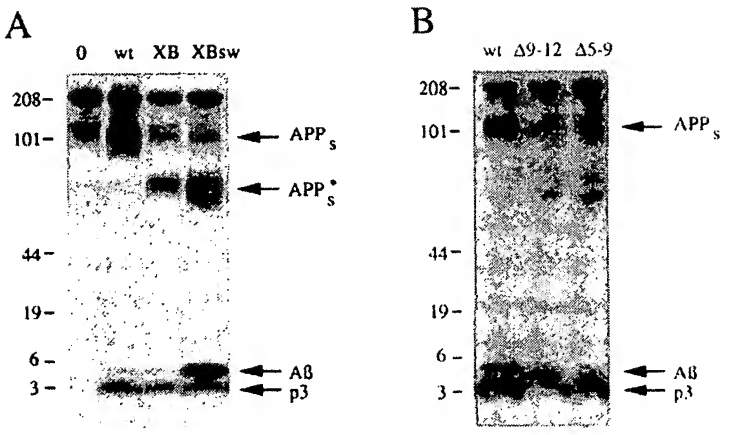

Figure 2. Antibody R1280 Immunoprecipitations of Conditioned Media from Metabolically Labeled Kidney 293 Cells Transiently Transfected with the Indicated Plasmids

(A) XhoI–BglII deletion constructs either without (XB) or with (XBsw) the Swedish double mutation. 0, untransfected; wt, transfected with wild-type βAPP$_{695}$. APP$_s$ marks the region of the gel in which conventional soluble forms of βAPP migrate. APP$_s$* marks the form of APP$_s$ that is shortened by ~30 kDa owing to the XB deletion. APP$_s$* is stronger in XBsw than in XB, presumably because of higher transfection efficiency.

(B) Aβ internal deletion constructs. In Δ5–9, Aβ and p3 run as distinct but very closely spaced bands. Radiosequencing of the upper band confirms that it has Aβ sequence (see Figure 3). Owing to the deletion of amino acids 9–12 and 5–9 of Aβ, the C-terminus of APP$_s$ is much less well recognized by R1280. The ~200 kDa proteins detected in all immunoprecipitations and the ~70 kDa protein in (B) are nonspecific proteins that are unrelated to βAPP (Haass et al., 1992b).

Radiosequencing of Aβ from Δ5–9 and Δ9–12 transfectants demonstrated that this is indeed the case (Figures 3A and 3C). For Aβ from both Δ5–9 and Δ9–12, major peaks of ^{3}H-phenylalanine were obtained at positions consistent with an Aβ start at Asp-1 (peak of radioactivity at cycle 4) and the deletion of 5 or 4 amino acids, respectively, between Phe-4 and Phe-19/20 (double peaks of radioactivity at cycles 14/15 or 15/16, respectively). As expected, p3 is not influenced by the deletions, and therefore both constructs show a p3 radiosequencing pattern very similar to the one previously established for wild-type βAPP (Haass et al., 1992b), with most of p3 starting at Leu-17 (Figures 3B and 3D). In summary, these data strongly suggest that the N-terminal cleavage generating Aβ can tolerate a decrease of distance of the peptide bond from the membrane.

Membrane Association of βAPP Is Required for β-Secretase Cleavage

Next, we asked whether βAPP must be membrane bound for Aβ production to occur. To address this question, five constructs were designed (Figure 4A), transiently transfected into 293 cells, and checked for Aβ production (Figure 4B). The wild-type βAPP$_{695}$ construct shows the expected bands of APP$_s$, Aβ, and p3 (Figure 4B). The construct STOP51 contains a stop codon at position 648, after the 51st amino acid from the Aβ start site (Figure 4A), thus including virtually the entire βAPP transmembrane domain. This construct produces Aβ and p3 comparably to the wild type (Figure 4B). However, products in the size range of APP$_s$ are markedly increased (see below). The construct STOP40 contains a stop codon at position 637, after the 40th amino acid from the Aβ start (Figure 4A).

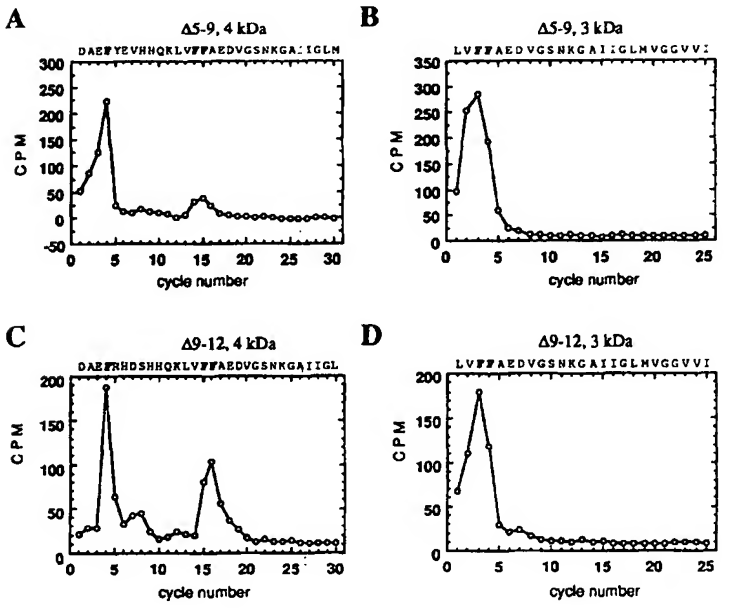

Figure 3. Radiosequencing of Aβ and p3 Peptides Secreted by 293 Cells Transfected with the Δ5–9 and Δ9–12 Mutations

The ^{3}H-phenylalanine radioactivity obtained at each cycle of the Edman chemistry is graphed for the 4 kDa (A and C) and 3 kDa (B and D) bands. The sequences above (A) and (C) are correct for the Δ5–9 and Δ9–12 molecules, respectively, i.e., they lack the deleted amino acids. The radiolabeled phenylalanines are indicated by bold letters (F) in each peptide sequence.

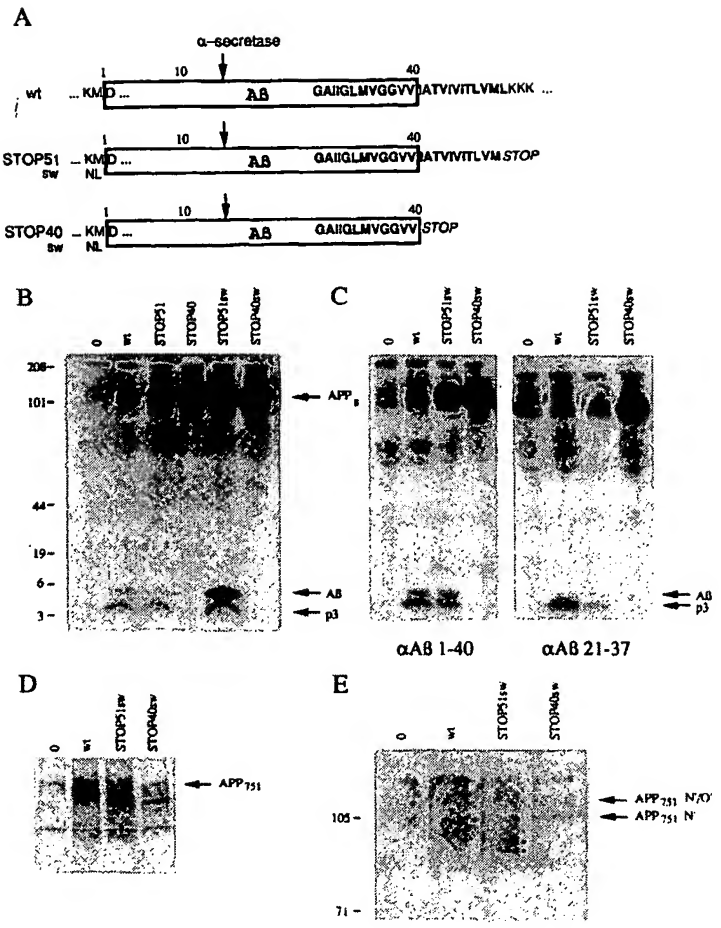

Figure 4. Membrane Insertion of βAPP Is Required to Generate Aβ

(A) βAPP schematic showing the C-termini of STOP51 and STOP40 constructs and the NL substitution in the constructs STOP51sw and STOP40sw. The amino acids of the transmembrane domain are indicated in bold letters.

(B) Antibody R1280 immunoprecipitations of conditioned media from metabolically labeled 293 cells transiently transfected with the indicated plasmids.

(C) Antibody 1280 ($Aβ_{1-40}$) and antibody 1963 ($Aβ_{21-37}$) immunoprecipitations of conditioned media from metabolically labeled 293 cells transfected with the indicated plasmids.

(D) Antibody B5 (to the midregion epitope $βAPP_{444-592}$) immunoprecipitations of total lysates of the cells used in (C). The principal endogenously expressed $βAPP_{751}$ precursor is indicated.

(E) Western blot of carbonate-extracted membrane preparations from kidney cells transfected with the indicated plasmids, using antibody B5. The two endogenous $βAPP_{751}$ bands are indicated. The $βAPP_{695}$ bands of the wild-type transfectant are marked (x). The lower molecular weight βAPP bands of the truncated STOP51sw construct are also marked (dots). This gel was run longer than (D) and therefore shows a clear separation of the N'- and the N'+O' glycosylated forms.

Thus, the resulting βAPP molecule terminates at the major cleavage position of γ-secretase (Figure 1) and would only need to undergo N-terminal cleavage to generate Aβ. This construct also produces very high amounts of material in the APP_s size range, but secretes neither Aβ nor p3 (Figure 4B). Similar results were obtained with the constructs STOP51sw and STOP40sw, which contain the Swedish mutation in addition to the STOP51 or STOP40 deletion, respectively (Figure 4B).

The fact that the constructs STOP51 and STOP51sw secrete Aβ while STOP40 and STOP40sw do not strongly suggests that only membrane-bound βAPP can function as a substrate for the Aβ N-terminal protease. Both truncation constructs lack the βAPP cytoplasmic domain, thus excluding the possibility that loss of this region alone causes the complete lack of Aβ generation in STOP40 and STOP40sw. The only difference between the constructs is the length of the transmembrane region they contain: STOP40 and STOP40sw contain only 12 amino acids of the predicted 24 amino acid transmembrane domain (Kang et al., 1987), whereas STOP51 and STOP51sw contain 23. It is therefore likely that the βAPP of STOP51 and STOP51sw occurs in considerable part as a membrane-bound molecule, whereas βAPP of STOP40 and STOP40sw does not. To test this prediction, we prepared a set of five dishes each from 293 cells that were either untransfected or transfected with $βAPP_{695}$, STOP51sw, or STOP40sw constructs. Two dishes from each set were metabolically labeled. The conditioned media of these two dishes were pooled, and one-half was immunoprecipitated with R1280, which is directed against $Aβ_{1-40}$ (Figure 4C). This precipitation showed again that Aβ and p3 are secreted at levels clearly above the endogenous background by wild type– and STOP51sw-transfected cells but not by STOP40sw-transfected cells. The untransfected cells showed very little material in the APP_s size range, the wild type–transfected cells show some APP_s, and both the STOP51sw- and STOP40sw-transfected cells have strong bands of similar intensity in that size range (Figure 4C). The second half of the conditioned media was immunoprecipitated with 1963, an antibody to Aβ residues 21–37 beyond the α-secretase site that recognizes Aβ and p3 but cannot precipitate APP_s ending at the α-secretase site (Figure 1). The Aβ and p3 precipitations from the various transfectants using antibody 1963 are similar to those obtained with R1280, with the p3 band being slightly more pronounced relative to the Aβ band (Figure 4C). However, a clear difference in immunoprecipitated material in the APP_s range is obtained with 1963. No material above background is precipitated by 1963 from wild type–transfected

cells, indicating that they do not release significant amounts of uncleaved βAPP from the membrane, as expected. In contrast, the STOP51sw-transfected cells clearly show some, and the STOP40sw-transfected cells show much more uncleaved βAPP that has been released intact from the membrane, and can thus be precipitated from the medium with 1963. The STOP40sw construct releases much higher amounts of uncleaved precursor, but produces neither Aβ nor p3; the latter, as an endproduct of the α-secretory pathway (Haass et al., 1993), is predicted to arise solely from membrane-associated βAPP (Sisodia, 1992).

If these differences in Aβ (and p3) secretion from the STOP51sw and STOP40sw molecules are indeed due to their different levels of retention in cell membranes, one would expect to find full-length STOP51sw and STOP40sw molecules in comparable amounts in total cell lysates, but only STOP51sw molecules in isolated membrane preparations. Figure 4D shows total cell lysates from the two metabolically labeled dishes immunoprecipitated with the βAPP midregion antibody, B5. All transfectants show the faint endogenous βAPP_{751} species. In addition, the wild-type transfectant has a major full-length band representing βAPP_{695} that migrates at a slightly lower molecular weight, as expected. The βAPP from the STOP51 deletion construct is 48 amino acids shorter than the wild-type molecule, and that from the STOP40 deletion construct is 59 amino acids shorter; bands of accordingly lower molecular weight were indeed detected in these extracts. This result indicates that all constructs produce βAPP molecules of the expected size in detectable amounts.

The remaining three dishes of each transfectant were used to prepare isolated carbonate-extracted membranes (see Experimental Procedures), which were then electrophoresed on SDS gels and immunoblotted with antibody B5 (Figure 4E). In all cases, the N′- and N′+O′-glycosylated forms of the endogenous βAPP_{751} were detected. In the βAPP_{695} wild-type transfectant, the N′+O′-glycosylated band of βAPP (marked by the upper "x" in Figure 4E) comigrates with the N-glycosylated form of the endogenous βAPP_{751}. In addition, the N-glycosylated form of the transfected βAPP_{695} migrating at a slightly lower molecular weight (marked by the lower "x") is clearly detectable. Two bands of lower molecular weight representing the N′- and N′+O′-glycosylated forms of the truncated STOP51sw molecule (marked by dots in Figure 4E) were detected in the membranes of STOP51sw cells. In contrast, only extremely faint bands, besides the endogenous βAPP_{751} background proteins, were detected in the STOP40sw deletion transfected cells. Together, Figures 4C–4E demonstrate that STOP40sw and STOP51sw are expressed and detectable in cell lysates. However, only STOP51sw is membrane inserted, and only STOP51sw undergoes the β-secretase cleavage necessary for Aβ production.

Differential Substrate Requirements for β-Secretase Cleavage of Wild-Type and Swedish βAPP_{695}

The data presented in Figure 4 seem to suggest that the whole βAPP C-terminus downstream of amino acid 648

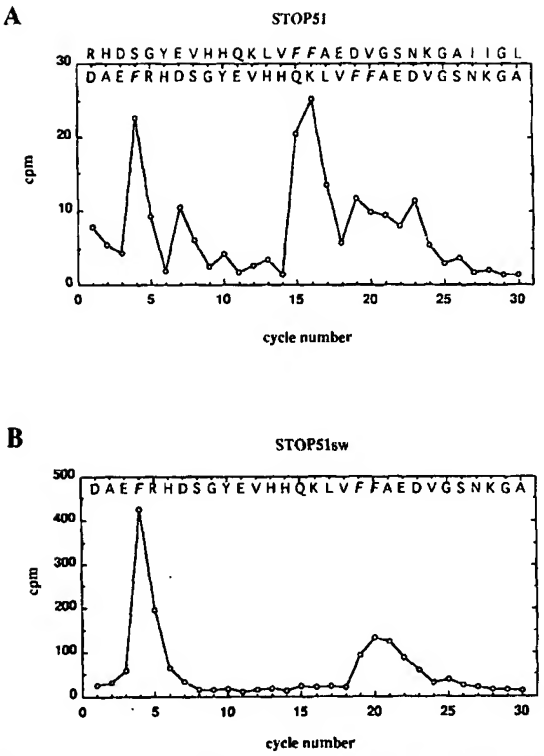

Figure 5. Radiosequencing of Peptides Secreted by Cells Transfected with the STOP51 or STOP51sw Constructs

The ^{3}H-phenylalanine-derived radioactivity obtained at each cycle of the Edman chemistry is graphed.

(A) Production of the 4 kDa peptide with STOP51. The upper line at the top of the graph shows the sequence of the predominant species starting at Arg-5, and the lower line shows the sequence of Aβ starting at Asp-1.

(B) Production of the 4 kDa peptide with STOP51sw. The sequence of the regular Asp-1 species is indicated.

(the endpoint of the STOP51 deletion) is dispensable for the production of Aβ. However, it has been shown that a deletion of the C-terminus of wild-type βAPP_{695} downstream from amino acid 653 (ΔC), including the reinternalization motif NPTY, decreases the amount of Aβ produced in 293 cells (Haass et al., 1993), and that deletion of the reinternalization sequence YENPTY alone has the same effect in CHO cells (Koo and Squazzo, 1994). Furthermore, whereas the ΔC mutation lowers the amount of Aβ starting at Asp-1, radiosequencing demonstrates that it significantly increases the amounts of Aβ-related peptides starting at Val(−3) (radioactive Phe at cycles 7, 22, and 23) and Arg-5 (radioactive Phe at cycles 15 and 16) relative to the amount of the Asp-1 Aβ form in the same cells (Haass et al., 1993). Radiosequencing of Aβ from STOP51-transfected 293 cells confirmed this observation (Figure 5A). Peptides starting at Arg-5 (radioactive Phe at cycles 15 and 16) become the predominant Aβ species. Surprisingly, this cytoplasmic deletion effect is not observed in Aβ from STOP51sw-transfected 293 cells (Figure 5B). Instead, a homogenous "Asp-1 only" N-terminal cleavage pattern is obtained. This pattern is typical for the effects

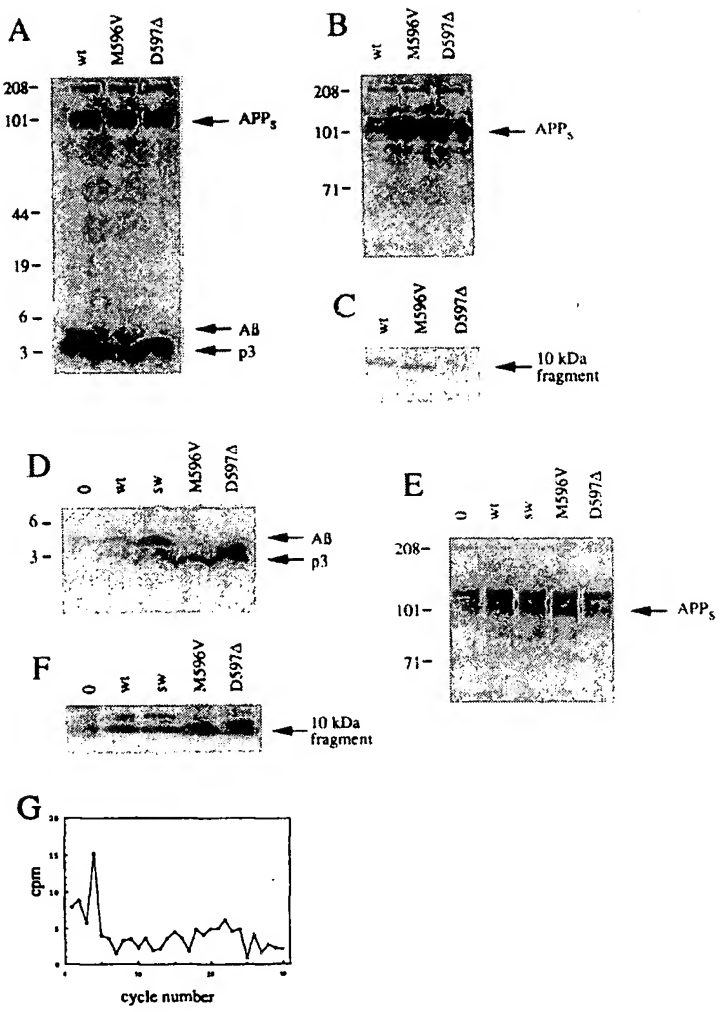

Figure 6. Immunoprecipitations of Conditioned Media and Cell Extracts from Metabolically Labeled Cells Transiently Transfected with the Indicated Plasmids

(A) R1280 immunoprecipitation of conditioned media from 293 transfectants.

(B) B5 immunoprecipitation of APP_s from conditioned media of 293 transfectants.

(C) C7 immunoprecipitation of C-terminal βAPP fragments from cell extracts of 293 transfectants.

(D) R1280 immunoprecipitation of conditioned media from SK-N-SH neuroblastoma transfectants.

(E) B5 immunoprecipitation of APP_s of conditioned media from SK-N-SH neuroblastoma cell transfectants. The APP_s band resulting from transfection with these βAPP_{695} wild-type or mutant constructs is marked. The band immediately above it is the endogenous APP_s.

(F) C7 immunoprecipitation of C-terminal βAPP fragments from cell extracts of SK-N-SH neuroblastoma cell transfectants.

(G) Radiosequencing of the 12 kDa band from extracts of SK-N-SH neuroblastoma cells transfected with Swedish mutant βAPP_{695} and precipitated with antibody C7. The ^{3}H-phenylalanine-derived radioactivity obtained at each cycle of the Edman chemistry is graphed.

For the M596V mutation, the amounts of the 10 kDa fragment and p3 seem slightly increased in 293 cells (C) and strongly increased in SK-N-SH cells (F), probably owing to a compensatory increase in α-secretase cleavage when β-secretase cleavage is inhibited.

of the Swedish mutation in full-length βAPP molecules and different from the heterogenous Aβ N-terminal cleavages of wild-type βAPP (Citron et al., 1994). Thus, whereas deletion of the entire cytoplasmic domain (or the YENPTY reinternalization signal alone) has a clear influence on β-secretase cleavage of wild-type βAPP, it has little or no effect on β-secretase cleavage of Swedish βAPP, consistent with the hypothesis that, for the latter molecule, β-secretase cleavage principally occurs before reinternalization of the precursor.

The N-Terminal Cleavage of Aβ Is Highly Sequence Specific

Previous experiments with transient and stable transfections of plasmids carrying the Swedish mutation suggest that amino acid substitutions at or near the N-terminal cleavage site can have profound effects on the amount of Aβ being produced in several types of cells (Citron et al., 1992; Cai et al., 1993). We therefore analyzed the effects of other amino acid substitutions in the region from Val(−3) (residue 594 of βAPP_{695}) to Ala-2 (residue 598) in transient transfections. The substitution constructs are abbreviated with the letter of the original amino acid, its position number, and the letter of the substituted amino acid; amino acid deletions are indicated by Δ. As an example of this set of experiments, Figure 6 shows all the βAPP derivatives obtained from the mutants M596V and D597Δ in both 293 cells and SK-N-SH human neuroblastoma cells. Conditioned media of the transfected cells were precipitated with the APP_s-reactive antibody B5, and all three constructs yielded APP_s proteins of the same size and in comparable amounts, whether expressed in 293 cells (Figure 6B) or SK-N-SH neuroblastoma cells (Figure 6E). However, precipitation of the conditioned media with the Aβ-specific antibody R1280 revealed striking differences among the constructs: while all three transfectants produced p3, which started at Leu-17 (data not shown) and is not observed in untransfected SK-N-SH cells, M596V produced no Aβ (Figures 6A and 6D). Likewise, D597Δ did not produce the 4 kDa Aβ species, but instead a ~3.8 kDa peptide (Figures 6A and 6D) that started at Phe-4 (radiosequencing data not shown). These results were ob-

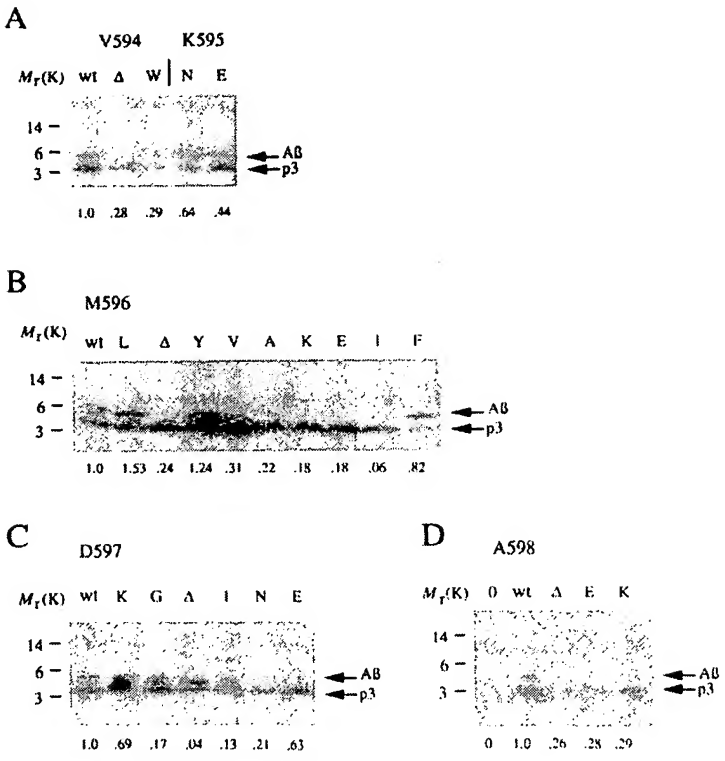

Figure 7. Antibody R1280 Immunoprecipitations of Conditioned Media from Metabolically Labeled 293 Cells Transiently Transfected with the Indicated Plasmids

(A) Mutations at V594 and K595. (B) Mutations at M596. (C) Mutations at D597. The amount of Aβ in D597K is overestimated because a slightly smaller band is not clearly separable for the quantitation. (D) Mutations at A598. An untransfected control just expressing the endogenous βAPP_{751} is also shown (lane 0). For each panel, the ratio of Aβ detected in conditioned medium to full-length βAPP detected in the cell lysate of each transfectant was determined by phosphorimaging and normalized to the wild-type ratio, which was set at 1.0. The ratios are shown below each panel. The ratios in (C), especially for D597K, may be overestimated, since shortened Aβ fragments overlap the 4 kDa range that was quantitated.

tained in both 293 cells (Figure 6A) and SK-N-SH cells (Figure 6D) after overnight labeling and were also seen after a 2 hr pulse labeling in the 293 cells (data not shown).

When cell extracts were precipitated with the C-terminal antibody C7, all transfected cells were found to overproduce membrane-bound, full-length βAPP (data not shown) and a 10 kDa C-terminal fragment that remains membrane-bound after release of APP_s into the medium (293 cells, Figure 6C; SK-N-SH cells, Figure 6F). An additional 12 kDa C-terminal fragment is observed in SK-N-SH neuroblastoma cells transfected with wild-type βAPP_{695} (Figure 6F). This band becomes much more pronounced in Swedish SK-N-SH transfectants, is barely detectable in the M596V transfectants, and is replaced by a shorter ~11.5 kDa band in the D597Δ transfectant (Figure 6F). A C-terminal fragment of similar size has been previously observed to be increased in M17 human neuroblastoma cells transfected with βAPP carrying the Swedish mutation and was thus hypothesized to be an immediate precursor of Aβ that has not yet undergone γ-secretase cleavage (Cai et al., 1993). Indeed, both the amounts and the size of this fragment correlate with the amounts and the size of Aβ in our various SK-N-SH transfectants (compare Figures 6D and 6F). Radiosequencing of the Swedish 12 kDa protein confirms that it indeed starts at Asp-1 (major peak of radioactive Phe in cycle 4; Figure 6G). It seems highly likely that the substitutions examined primarily influence the N-terminal cleavage of Aβ and not other βAPP proteolytic processing pathways, because we did not observe any substantial differences in cellular full-length βAPP (data not shown) or decreases in total APP_s (Figures 6B and 6E).

We next set out to test an extensive panel of N-terminal substitutions for their effect on Aβ production in 293 cells. For each substitution, the R1280 immunoprecipitates of Aβ and p3 are shown in Figure 7. The relative ratios of Aβ to full-length βAPP, determined by phosphorimaging and set as 1.0 for the wild type in each panel, are indicated (Figure 7). All of the substitution mutants produced full-length βAPP and APP_s (data not shown). Likewise, p3 was secreted by all of the mutants analyzed, although in varying amounts. In contrast, Aβ production was markedly affected by most substitutions. Changes at V594 strongly decreased Aβ production, whereas no clear effect was observed for two substitutions for K595. Several substitutions at the p1 position M596 were analyzed. The previously described M596L substitution of the Swedish mutation (Citron et al., 1992) led to an increase in Aβ. Of all the other substitutions, only the hydrophobic, bulky residues F and Y did not lead to a strong decrease in Aβ (Figure 7B). The Aβ 4 kDa species was virtually eliminated by all the D597 substitutions. Only D597E still showed the original Aβ band (Figure 7C). However, in all these D597 substitutions, intermediate bands of ~3.8 kDa appeared that were not observed from wild-type constructs (Figure 7C), as shown above for D597Δ (Figure 6D). Substitutions of A598 also had a marked inhibitory effect on Aβ production (Figure 7D). In summary, almost all of the single amino acid substitutions at and immediately around the N-terminal cleavage site markedly decreased Aβ production.

Discussion

In this work, we have used human 293 cells to establish the substrate requirements of proteases that cleave βAPP at the N-terminus of Aβ. We show that three βAPP mutants producing strong, but distinct, effects on Aβ production in this cellular system give essentially identical results in human SK-N-SH neuroblastoma cells. These data suggest that mutations around the Met–Asp cleavage site may lead to similar changes in Aβ production in other neural and nonneural cell types. Our findings demonstrate that the region defined by residues 590–600 (βAPP_{695} numbering) is sufficient for recognition and efficient cleavage of the precursor by the Aβ N-terminal protease(s). When the cleavage site is moved 5 amino acids closer to the membrane, there is no effect on cleavage, as demonstrated by N-terminal sequencing of the product. This result suggests that the protease(s), designated β-secretase(s), recognizes the primary structure around the cleavage site independently of its distance from the membrane, a property which is the opposite of that postulated for α-secretase (Sisodia, 1992). The fact that β-secretases do not require a fixed distance to the membrane in order to cleave suggests that the proteases themselves are not membrane anchored. However, they can only act on membrane-bound βAPP molecules in intact cells, because otherwise the STOP40 and STOP40sw forms of βAPP would give rise to Aβ, as do the STOP51 and STOP51sw constructs, which also lack the cytoplasmic domain but contain most of the transmembrane domain. The fact that only membrane-bound βAPP is N-terminally cleaved in vivo implies that Aβ is very unlikely to be produced extracellularly from larger secreted N- or C-terminal fragments of full-length βAPP, at least not by the same proteases that produce Aβ in intact cells.

Two lines of indirect evidence suggest that more than one protease can generate the N-terminus of Aβ: first, the wild-type βAPP molecule can be processed into Aβ-related peptides starting at different positions in a cell type–dependent manner; second, the N-terminal sequence heterogeneity of Aβ peptides produced in 293 cells suggests the action of more than one enzyme, particularly in view of our finding here that the Met–Asp cleaving enzyme is highly sequence specific. The results of substitutions at the p1 position, in which only the large hydrophobic residues Met, Leu, Phe, and Tyr allowed cleavage, are consistent with the primary cleavage at Asp-1 being produced by a chymotryptic-like proteinase. Based on our Aβ sequencing data, this enzyme would be the only one capable of creating the Aβ N-terminus in fibroblasts, would be the predominant enzyme in 293 cells, and would be virtually absent from the normal Aβ production pathway in Madin–Darby canine kidney cells (in which Aβ begins primarily at Arg-5). βAPP substrates with mutations around the regular N-terminal cleavage site could then be recognized by other proteases with different specificities. This would explain the 3.8 kDa peptides that arise after virtually any substitution at D597. The situation is complicated further by the fact that not all enzymes capable of cleaving the N-terminus of Aβ need necessarily be located in the same subcellular compartment. It has recently been demonstrated that one site of Aβ generation is within an early endosome following reinternalization of βAPP from the cell surface (Koo and Squazzo, 1994). However, βAPP molecules such as STOP51, in which the reinternalization signal is deleted, still produce Aβ, albeit in reduced amounts and with increased levels of Aβ-related peptides starting at positions other than Asp-1.

Most of the single amino acid substitutions we tested substantially decreased or eliminated Aβ production. We believe this result is due to reduced cleavage at the Aβ N-terminus rather than to decreased stability of the peptide or destruction of an epitope essential for the immunoprecipitation, because substitutions at V594 and M596 in front of the cleavage site would not influence antibody recognition or stability of a peptide starting at D597. Furthermore, the loss of the 4 kDa peptide in D597Δ was also observed in a short-pulse experiment, arguing against Aβ destabilization by substitution as a cause for its reduction in the D597 mutants. The fact that R1280 precipitates p3 shows that it does not recognize solely epitopes at the N-terminus of Aβ. This makes it unlikely that single amino acid substitutions at the Aβ N-terminus would completely block R1280 binding and thereby account for the disappearance of the 4 kDa band.

Our data underscore the previous conclusion that p3 arises from a different processing pathway than Aβ (Haass et al., 1993). The fact that only the Swedish mutation, with its M596L substitution, clearly increases Aβ production is consistent with the assumption that the Leu–Asp bond is a much better substrate than the wild-type Met–Asp bond for an Asp-1 cutting protease that cleaves βAPP during its transport to the cell surface. This cleavage would happen prior to the reinternalization that appears to be necessary for the principal Asp-1 cleavage event in the wild-type substrate. Such a model would explain why the cleavage pattern of Swedish βAPP is not affected by C-terminal truncations like STOP51sw that delete the internalization signals. Because none of the other mutants we examined besides M596L led to such a strong increase in Aβ production, we speculate that any other polymorphisms around the cleavage site would also be phenotypically silent, making it unlikely that additional FAD mutations which cause increased Aβ production will be mapped to the N-terminus of Aβ.

The present investigation describes several properties of the β-secretase enzymes that produce Aβ in living human cells and also allows two general conclusions about the C-terminal γ-secretase cleavage. First, the correlation of the amount of the 12 kDa C-terminal βAPP fragment starting at Asp-1 with the amount of Aβ, and the correlation of the amount of the respective 11.5 kDa fragment produced by D597Δ with the amount of the shortened Aβ-type peptide starting at Phe-4 in SK-N-SH cells, confirm the previous suggestion of Cai et al. (1993) that the 12 kDa fragment can serve as the immediate precursor of Aβ and indicate that β-secretase cleavage occurs before γ-secretase cleavage. The 12 kDa molecule has not been observed, however, in transfected 293 cells (Figure 6C) (Citron et al., 1992) or in primary skin fibroblasts from patients

carrying the Swedish FAD mutation (Citron et al., 1994), suggesting that there are cell type differences in the rate at which the 12 kDa molecule undergoes γ-secretase cleavage. Second, because Aβ is still produced from constructs like STOP51sw, γ-secretase does not require the cytoplasmic domain of βAPP to recognize and cleave the C-terminus of Aβ.

Finally, our description of the substrate specificity of the protease has important practical consequences. For example, our data suggest that βAPP recombinant derivatives or synthetic peptides used to identify and characterize this protease must at least include Val-594 to Ala-598. Furthermore, one can now design control substrates with substitutions like M596V, which should not be cleaved well by the candidate protease. However, as we have shown here, only membrane-bound βAPP undergoes N-terminal cleavage in vivo. If this need for membrane anchoring reflects a requirement of the protease rather than a requirement for substrate compartmentalization and targeting, then βAPP derivatives and synthetic peptides that are not membrane bound should not be recognized by the protease for which they are designed. Therefore, the use of synthetic peptides or recombinant fragments in solution to screen for β-secretase might well lead to the isolation of irrelevant enzymes. These various considerations emphasize the importance of acquiring in vivo data in intact human cells when searching for physiologically relevant proteases.

Experimental Procedures

Transfection, Metabolic Labeling, Preparation of Total Cell Lysates, Immunoprecipitation, and Protein Radiosequencing

Details of these methods have been described previously (Haass et al., 1992b). Briefly, subconfluent 10 cm dishes of human embryonic kidney 293 cells or SK-N-SH human neuroblastoma cells were transiently transfected with 20 μg of supercoiled plasmid DNA using Lipofectin (GIBCO/BRL) as described by the manufacturer. About 48 hr after transfection, cells were metabolically labeled with [^{35}S]methionine overnight. Immunoprecipitations were performed as described (Haass et al., 1992b). Immunoprecipitated APP$_s$ was separated on a 10% SDS–polyacrylamide gel, whereas precipitates of Aβ and p3 from media and precipitates of cell extracts were separated on 10%–20% Tris–Tricine gels. Autofluorography was carried out as described (Haass et al., 1992b). Bands were quantitated by phosphorimaging. Radioactive sequencing of the 4 kDa (Aβ) and 3 kDa (p3) peptides was performed after R1280 immunoprecipitation of transiently transfected cells metabolically labeled with L-[2,3,4,5,6-^{3}H]phenylalanine as described (Haass et al., 1992b).

Antibodies Used for Immunoprecipitation

The polyclonal antibody C7 (Podlisny et al., 1991) is directed against the last 20 amino acids of the cytoplasmic tail of βAPP. This antibody immunoprecipitates N′- and N′+O′-glycosylated full-length βAPP and the 10 kDa fragment. The affinity-purified polyclonal antibody B5 (Oltersdorf et al., 1990) was raised to a recombinantly expressed protein of βAPP$_{644–592}$ and immunoprecipitates APP$_s$ and N′- and N′+O′-glycosylated full-length βAPP. The polyclonal antibody R1280 (Tamaoka et al., 1992) was raised to synthetic Aβ$_{1–40}$. This antibody immunoprecipitates Aβ, p3, and small, variable amounts of APP$_s$ from media of tissue culture cells (Haass et al., 1992b). The polyclonal antibody 1963 (Haass et al., 1992b) was raised to synthetic Aβ$_{21–37}$.

Plasmid Constructions

All constructs used in this paper are derivatives of pCMV695, a plasmid carrying βAPP$_{695}$ cDNA under control of the CMV promoter (Seikoe et al., 1988). Constructs containing single amino acid substitutions were designed by replacing the 26 bp BglII–EcoRI fragment of βAPP$_{695}$ with annealed oligonucleotides in which codon 594, 595, 596, 597, or 598 was changed to encode the mutant amino acid: GTG was changed to TGG in V594W and deleted in V594Δ; AAG was changed to AAT in K595N and to GAG in K595E; ATG was deleted in M596Δ and changed to CTG in M596L, to TAT in M596Y, to GTG in M596V, to GCC in M596A, to AAG in M596K, to GAG in M596E, to ATC in M596I, and to TTC in M596F; GAT was deleted in D597Δ and changed to AAA in D597K, to GGA in D597G, to ATC in D597I, to AAC in D597N, and to GAA in D597E; GCA was deleted in A598Δ and changed to GAG in A598E and to AAG in A598K. The plasmids XB and XBsw were engineered by deleting the XhoI–BglII fragment from pCMV695 and pCMV695 KM-NL (Citron et al., 1992), respectively, and replacing it with the annealed oligonucleotides TCGAGACACCTGGGGA and GATCTCCCCAGGTGTC. The plasmids Δ5–9, Δ9–12, Stop40, and Stop51 were generated by site-directed mutagenesis (Kunkel, 1985) of βAPP$_{695}$ cDNA using the oligonucleotides: CCAATTTTTGATGATGAACTTC ATAGAATTCGTAATCATGGTCAT, GAACACCAATTTTTGATGTGAGTCATGTCGGAATTCGTAATC, GATCACTGTCGCTTAGACAACACCGCC, and CTGTTTCTTCTTCTAGATAACCAAGGTGATGAC, respectively. The constructs STOP51sw and STOP40sw were engineered by replacing the wild-type EcoRI–SpeI fragment of the Swedish mutant construct βAPP$_{695}$ KM-NL (Citron et al., 1992) with the EcoRI–SpeI fragment from STOP51 and STOP40, respectively. Except for XB, all mutations were verified by DNA sequencing.

Preparation of Isolated Membranes

A postnuclear supernatant was prepared as described previously (Haass et al., 1992a) and pelleted to remove cytoplasmic proteins by a high speed spin for 1 hr at 50,000 rpm (233,000 g) at 4°C in a Beckman 55Ti rotor. Isolated membranes were carbonate extracted as described (Fujiki et al., 1982). The homogenate was pelleted as described above. For each sample, 15 μg of total protein were loaded on a 10% SDS–polyacrylamide gel. Transfer and immunoblotting were performed as described (Haass et al., 1992a).

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BIOCHEMISTRY

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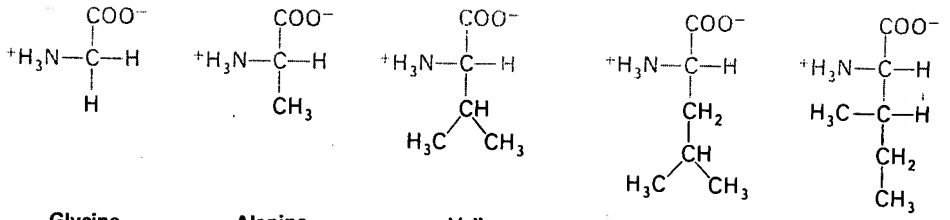


Figure 2-8
Amino acids having aliphatic side chains.

Let us look at this repertoire of amino acids. The simplest one is *glycine*, which has just a hydrogen atom as its side chain (Figure 2-8). *Alanine* comes next, with a methyl group as its side chain. Larger hydrocarbon side chains (three and four carbons long) are found in *valine*, *leucine*, and *isoleucine*. These larger aliphatic side chains are *hydrophobic*—that is, they have an aversion to water and like to cluster. As will be discussed later, the three-dimensional structure of water-soluble proteins is stabilized by the coming together of hydrophobic side chains to avoid contact with water. The different sizes and shapes of these hydrocarbon side chains (Figure 2-9) enable them to pack together to form compact structures with few holes.

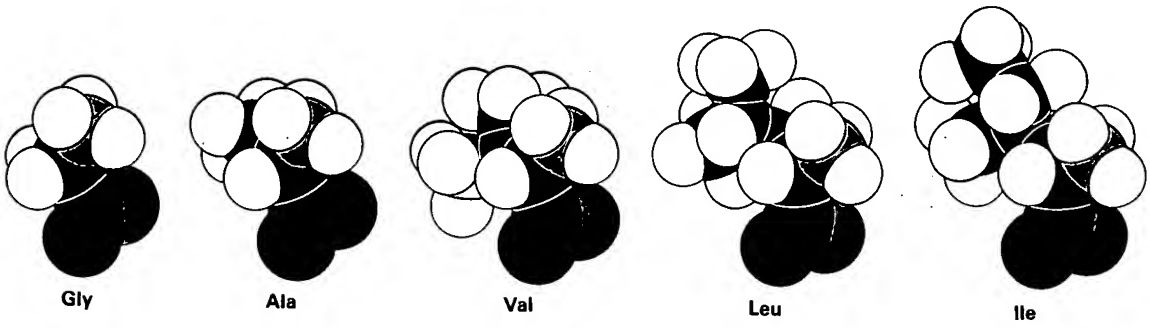


Figure 2-9
Models of aliphatic amino acids

Proline also has an aliphatic side chain but it differs from other members of the set of twenty in that its side chain is bonded to both the nitrogen and α-carbon atoms. The resulting cyclic structure (Figure 2-10) markedly influences protein architecture. Proline, often found in the bends of folded protein chains, is not averse to being exposed to water. Note that proline contains a secondary rather than a primary amino group, which makes it an *imino* acid.

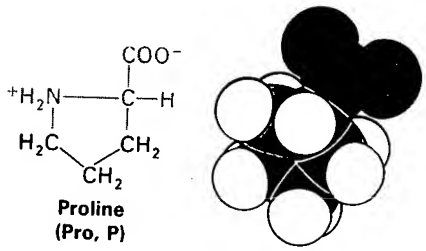


Figure 2-10
Proline differs from the other common amino acids in having a secondary amino group.

Three amino acids with *aromatic side chains* are part of the fundamental repertoire (Figure 2-11). *Phenylalanine*, as its name indicates, contains a phenyl ring attached to a methylene (—CH_2—) group. *Tryptophan* has an indole ring joined to a methylene group; this side chain contains a nitrogen atom in addition to carbon and hydrogen atoms.

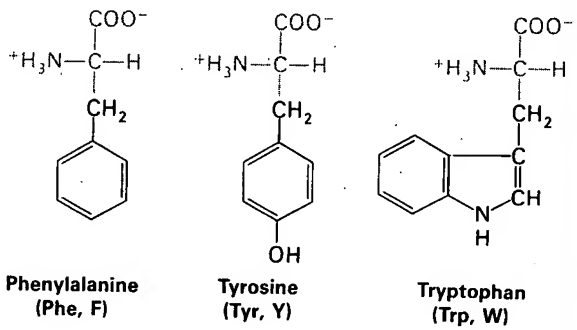


Figure 2-11
Phenylalanine, tyrosine, and tryptophan have aromatic side chains.

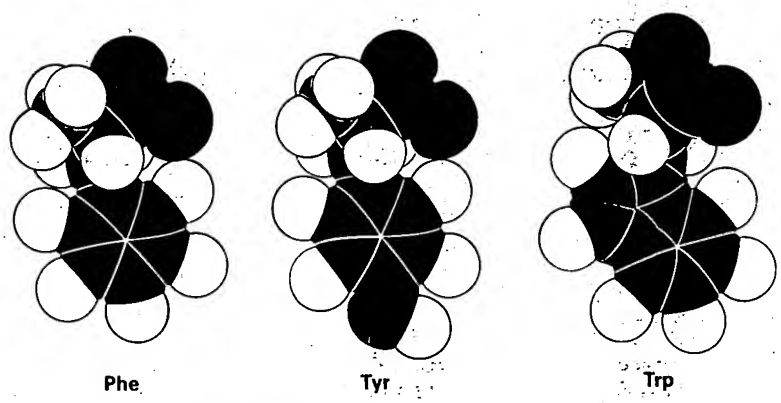


Figure 2-12
Models of the aromatic amino acids.

Phenylalanine and tryptophan are highly hydrophobic. The aromatic ring of *tyrosine* contains a hydroxyl group, which makes tyrosine less hydrophobic than phenylalanine. Moreover, this hydroxyl group is reactive, in contrast with the rather inert side chains of all the other amino acids discussed thus far. The aromatic rings of phenylalanine, tryptophan, and tyrosine contain delocalized pi-electron clouds that enable them to interact with other pi-systems and to transfer electrons.

A *sulfur atom* is present in the side chains of two amino acids (Figure 2-13). *Cysteine* contains a sulfhydryl group (—SH) and *methionine* contains a sulfur atom in a thioether linkage (—S—CH_3). Both of these sulfur-containing side chains are hydrophobic. The sulfhydryl group of cysteine is highly reactive. As will be discussed shortly, cysteine plays a special role in shaping some proteins by forming disulfide links.

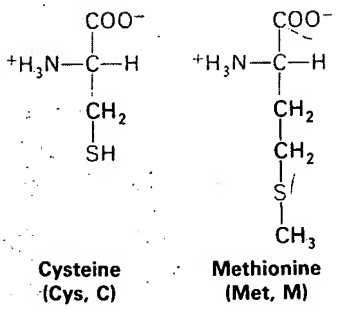


Figure 2-13
Cysteine and methionine have sulfur-containing side chains.

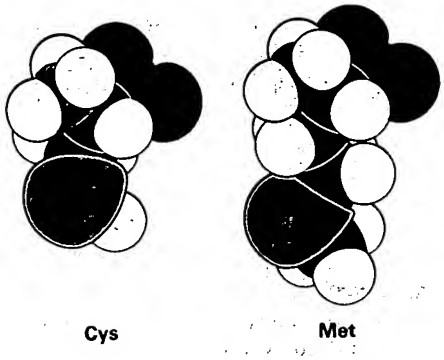


Figure 2-14
Models of cysteine and methionine.

Two amino acids, *serine* and *threonine*, contain aliphatic *hydroxyl groups* (Figure 2-15). Serine can be thought of as a hydroxylated version of alanine, and threonine as a hydroxylated version of valine. The hydroxyl groups on serine and threonine make them much more *hydrophilic* (water-loving) and *reactive* than alanine and valine. Threonine, like isoleucine, contains two centers of asymmetry. All other amino acids in the basic set of twenty, except for glycine, contain a single asymmetric center (the α-carbon atom). Glycine is unique in being optically inactive.

We turn now to amino acids with very polar side chains, which render them highly *hydrophilic*. *Lysine* and *arginine* are *positively charged* at neutral pH. *Histidine* can be uncharged or positively charged, depending on its local environment. Indeed, histidine is often found in the active

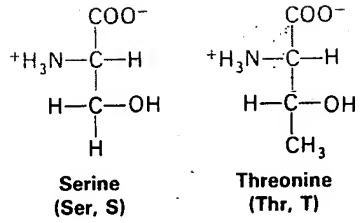


Figure 2-15
Serine and threonine have aliphatic hydroxyl side chains.

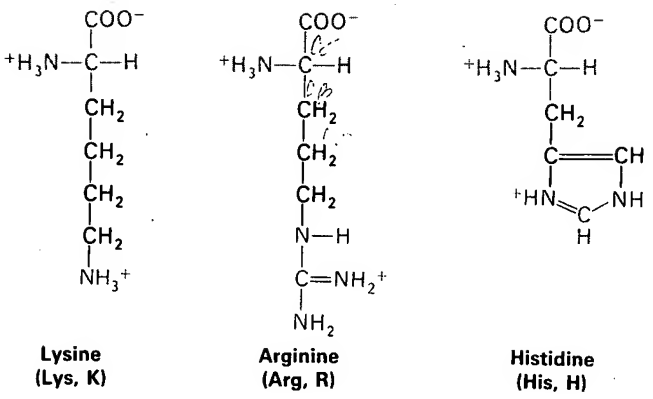


Figure 2-16
Lysine, arginine, and histidine have basic side chains.

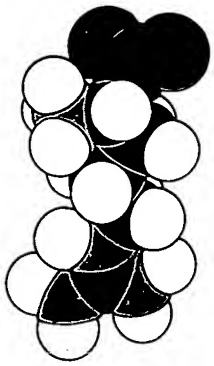


Figure 2-17
Model of arginine. The planar outer part of the side chain, consisting of three nitrogens bonded to a carbon atom, is called a guanidinium group.

sites of enzymes, where its imidazole ring can readily switch between these states to catalyze the making and breaking of bonds. These *basic amino acids* are depicted in Figure 2-16. The side chains of arginine and lysine are the longest ones in the set of twenty.

The repertoire of amino acids also contains two with *acidic side chains*, *aspartic acid* and *glutamic acid*. These amino acids are usually called *aspartate* and *glutamate* to emphasize that their side chains are nearly always negatively charged at physiological pH (Figure 2-18). Uncharged derivatives of glutamate and aspartate are *glutamine* and *asparagine*, which contain a terminal amide group in place of a carboxylate.

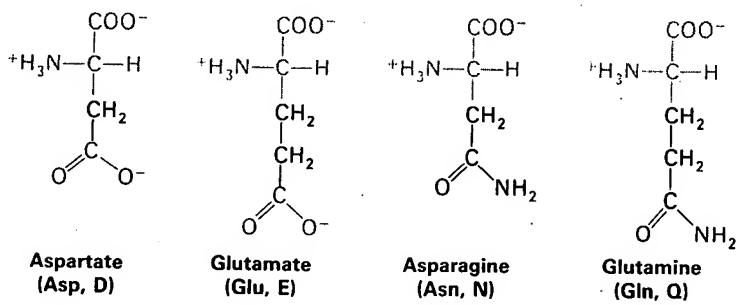


Figure 2-18
Acidic amino acids (aspartate and glutamate) and their amide derivatives (asparagine and glutamine).

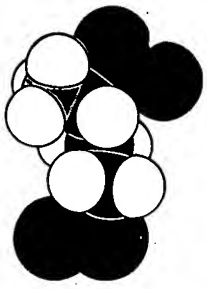
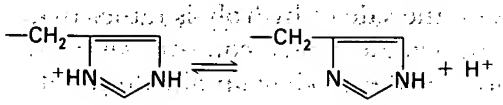
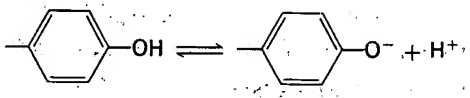
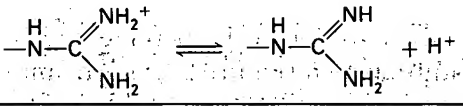


Figure 2-19
Model of glutamate.

Seven of the twenty amino acids have readily ionizable side chains. Equilibria and typical pK_a values for ionization of the side chains of arginine, lysine, histidine, aspartic and glutamic acids, cysteine, and tyrosine in proteins are given in Table 2-1. Two other groups in proteins, the terminal α-amino group and the terminal α-carboxyl group, can be ionized.

Amino acids are often designated by either a three-letter abbreviation or a one-letter symbol to facilitate concise communication (Table 2-2). The abbreviations for amino acids are the first three letters of their names, except for tryptophan (Trp), asparagine (Asn), glutamine (Gln), and isoleucine (Ile). The symbols for the small amino acids are the first letters of their names (e.g., G for glycine and L for leucine); the other symbols have been agreed upon by convention. These abbreviations and symbols are an integral part of the vocabulary of biochemists.

Table 2-1
pK values of ionizable groups in proteins

Group	*Acid ⇌ base + H⁺*	*Typical pK**
Terminal carboxyl	$-COOH \rightleftharpoons -COO^- + H^+$	3.1
Aspartic and glutamic acid	$-COOH \rightleftharpoons -COO^- + H^+$	4.4
Histidine		6.5
Terminal amino	$-NH_3^+ \rightleftharpoons -NH_2 + H^+$	8.0
Cysteine	$-SH \rightleftharpoons -S^- + H^+$	8.5
Tyrosine		10.0
Lysine	$-NH_3^+ \rightleftharpoons -NH_2 + H^+$	10.0
Arginine		12.0

*pK values depend on temperature, ionic strength, and the microenvironment of the ionizable group.

Table 2-2
Abbreviations for amino acids

Amino acid	*Three-letter abbreviation*	*One-letter symbol*
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Gurney et al.	)	For: Alzheimer's Disease Secretase, APP Substrates Therefor, and Uses Thereof
Examiner: S. Turner	)	
Group: 1647	)	

DECLARATION OF MICHAEL BIENKOWSKI, Ph.D.
PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
Washington, DC 20231

Sir:

I, Michael Jerome Bienkowski, Ph.D., hereby declare as follows:

I. Introduction

1. I am a co-inventor of Asp2 subject matter claimed in various patent applications filed by Pharmacia & Upjohn. I make this declaration to provide information to the Patent Office that may be relevant to patent issues relating to enzymatically active, "transmembrane-deleted" forms (ΔTM) of the Asp2 protein and polynucleotides which encode such protein. When I refer to "I" or "we" in this declaration, I mean me and/or my co-inventors and/or people working under our direction at Pharmacia & Upjohn.

2. The term "Asp2" is the name that we gave to aspartyl protease polynucleotides and polypeptides that we isolated and described in the patent applications. At least two human and one murine form of Asp2 are taught in the patent applications. Through experiments described in the patent applications we demonstrated that Asp2 exhibits proteolytic activity towards amyloid precursor protein (APP) involved in processing APP into amyloid beta (Aβ), a peptide implicated in Alzheimer's Disease pathology.

II. Cloning of Asp2 and Identifying the Asp2 transmembrane domain

3. My co-inventors and I performed and/or directed experiments which resulted in the identification and cloning of human Asp2 cDNAs. Our earliest experiments did not immediately yield full-length Asp2 cDNAs. We first obtained and sequenced two

partial clones denoted as clone 4386993 (hereinafter '438) and clone 2696295 (hereinafter '269). As explained in our patent applications, Clone '438 contains additional codons sequence at its 5' end relative to clone '269, but Clone '269 contains 25 additional codons (75 basepairs) as an internal insertion relative to Clone '438. (These 25 codons represent the difference between the long and short forms of full length human Asp2 in Figures 2 and 3 of the patent applications.)

4. After we sequenced the '438 and '269 clones we aligned the sequences with sequences of other aspartyl proteases as part of our analysis of them. From these alignments and other analysis we deduced that these sequences were incomplete cDNA sequences that were truncated at the 5' end (the amino-terminus of the encoded polypeptide). Computer-aided analysis of the predicted amino acid sequences indicated that the predicted amino acid sequence encoded by both '438 and '269 contained the DTG/DSG sequences indicative of the aspartyl protease active site, and were complete to the carboxyl-terminus of the encoded polypeptide.

5. By analyzing the partial Asp2 sequence from the '438 and '269 clones described in paragraph 3, we deduced that Asp2 contained a transmembrane domain. Our U.S. Provisional Application No. 60/101,594, filed September 24, 1998, describes the analysis as follows:

> Routine computer-aided analysis of the predicted amino acid sequence of Hu-Asp2a and Hu-Asp2(b) for secondary structure motifs resulted in detection of a predicted transmembrane domain in each polypeptide, which corresponds to Hu-Asp2(a) amino acid residues 367-392 of SEQ ID NO: 4, and of the sequence given in Figure 2, and to Hu-Asp 2(b) amino acid residues 392-417 of SEQ ID NO: 6, and of the sequence given in Figure 3.
>
> (See U.S. Provisional Application No.60/101,594 at p. 20.)

As I explain in greater detail below, the stated location of the transmembrane sequences (367-392) and (392-417), through an inadvertent error, do not correspond to the transmembrane regions of the full length human Asp2(a) and Asp2(b) proteins shown in the Figures, and standing alone, these numbers would not serve as a basis for identifying the transmembrane region of the human Asp2 sequences. However, our routine computer-aided

analysis did, in fact, permit us to identify the Asp2 transmembrane region, and a molecular biologist of ordinary ability who read the application and (through the guidance of the application) performed his/her own routine computer-aided analysis would have identified the correct location of the transmembrane region in our Asp2 sequences.

6. Through our continued research we ultimately cloned additional 5' (amino terminal) cDNA sequence for the two human Asp2 enzyme isoforms. As reported in our patent applications, the longer full length human Asp2 cDNA has 501 codons.[1] (Figure 3 of the patent applications.) As correctly reported in our 1999 patent applications, the transmembrane domain of this Asp2 clone spans approximately residues 455 to 477 of the full length Asp2 sequence.

7. Looking back, I believe that the inadvertent error in the 60/155,493 application occurred because our research team had performed some of the routine computer-aided analysis on a partial Asp2 sequence from the '438 clone, and reported the data from this analysis for the full length Asp2 clone in the patent application. The analysis of the partial sequence from the '438 clone indicated that the transmembrane domain corresponded approximately to residues 367-392 *of the partial sequence.* (See Exhibit A hereto, which is a computer-assisted analysis of Asp2 (clone '438) sequence for possible transmembrane domains, performed prior to September 24, 1998, which indicates a likely TM region at about 367-392 of the sequenced analyzed.) I believe that the numbers from this analysis *of the '438 partial sequence* were reported in the 60/155,493 application for the *full length* short form (Figure 2) of human Asp2.[2] Since the patent application reported the full length Asp2 sequences, the numbers that were generated using the '438 clone partial sequence should have been adjusted upward for the patent application, to account for the extra codons at the beginning of the full length clone that were missing from the '438 clone partial sequence

---

[1] An Asp2 splice variant described in our patent application has 476 codons by virtue of the internal deletion of 25 codons described above in paragraph 3. (Figure 2 of the patent applications.) As reported correctly our 1999 patent applications, the transmembrane domain of this sequence corresponds approximately to residues 430-452.

[2] An upward adjustment of these numbers (by 25 codons) was used for the long form of Asp2 (Figure 3).

analyzed. But, through inadvertent error when preparing the patent application, this adjustment was not made.

8. In my opinion, this error would have been apparent to an average scientist in the field who evaluated the application, as would the proper correction of the error. In particular, it is commonly understood by molecular biologists that a transmembrane domain is characterized by a stretch of about 20-25 mostly hydrophobic amino acids. When a biologist read the application's teaching that Asp2 had a transmembrane domain near the carboxy-terminus and then examined the sequence to look for that transmembrane domain, it would have been readily apparent that the transmembrane domain was at about residues 455-477 (of Figure 3), and not residues 392-417.

III. **Invention-related activity for Asp2 ΔTM polynucleotides and polypeptides.**

9. The attorneys for Pharmacia & Upjohn have asked me to authenticate and discuss certain documents relating to our Asp2 invention.

10. Exhibit B hereto comprises excerpts from our U.S. Provisional Application No. 60/101,594. These excerpts establish that, on or before our filing date of September 24, 1998, we had possession of two human Asp2 cDNA and deduced Asp2 amino acid sequences (Figures 2 and 3) and determined various Asp2 structural features, including the presence of a transmembrane domain. It shows that we contemplated vectors and host cells for recombinant production of Asp2 polypeptides and enzymatically active polypeptide fragments (see, e.g., pp. 4, 5, and 9), and that we contemplated Asp2 antibodies (see, e.g., pp. 4, 12.) It shows that we contemplated expression of Asp2 in a variety of expression systems, including prokaryotes such as *E. coli* (pp. 9 and 10), yeasts such as *S. cerevisiae* (pp. 9, 11), and higher eukaryotes such as insect cell systems and mammalian systems, including COS cells, CHO cells, and human cells (see, e.g., pp. 9, 11-12).

11. Exhibit C hereto is a copy of a page from a Pharmacia & Upjohn interoffice memo from prior to our September 24, 1998, filing date, containing a report on the Human Asp2 project. Among other things, this excerpt shows that, prior to September 24,

1998, we had engineered the Asp2 open reading frame (ORF) from the '438 and '269 clones to remove the transmembrane domains, and that we had inserted these ΔTM constructs into an *E. coli* expression vector pQE30.

12. Exhibit D hereto are copies of pages from a Pharmacia & Upjohn laboratory notebook. These pages establish that, prior to December 31, 1998, we had made a human Asp 2 ΔTM construct containing the DNA sequence coding for human Asp2 amino acids 1-454 (long form shown in Figure 3 of patent applications) in a baculovirus expression vector pVL 1393 (hu Asp 2 ΔTM pVL 1393) for expression in SF9 insect cells. This construct was sent for sequencing and the sequence was confirmed. Exhibit E hereto are copies of pages from a Pharmacia & Upjohn laboratory notebook. These pages establish that after December 31, 1998, we had made similar constructs with 6-histidine tags to facilitate protein purification.

13. Exhibit F hereto are copies of pages from Pharmacia & Upjohn laboratory notebooks which show that, prior to March 26, 1999, we had expressed human Asp2 ΔTM protein (without ß secretase enzyme activity) in *E. Coli* to make antibodies for use in testing of recombinant expression of human Asp2 ΔTM in other cell types.

14. Exhibit G hereto are copies of pages from a Pharmacia & Upjohn laboratory notebook which show that, prior to March 26, 1999, we had made, isolated, and scaled-up preparations of viral plaques for production of a human Asp2 ΔTM construct in SF9 insect cells.

15. Exhibit H hereto contains copies of pages from a Pharmacia & Upjohn laboratory notebook which show that, after December 31, 1998, and prior to June 15, 1999, and prior to September 23, 1999, the scale-up results from SF9 were analyzed. Exhibit I are copies of a Pharmacia and Upjohn laboratory notebook showing a gel depicting the results of such analysis. A clean band of human Asp2 ΔTM expressed protein was identified by Western blot as shown in the notebook. This band is believed to contain active human ΔTM Asp2 1-454 protein expressed in the SF9 system.

16. Exhibit J are copies of pages from a Pharmacia & Upjohn laboratory notebook showing that, after March 26, 1999, but prior to September 23, 1999, we excised the 1-454 Asp2 ΔTM coding segment from the pVL 1393 vector described above, inserted it into PIZ vector, and expressed this Asp2 ΔTM construct in High Five Cells. We tested this recombinant human Asp2 ΔTM protein and showed that it retained human Asp2 enzymatic activity. This work is also generally described in the patent applications that we filed on September 23, 1999, including PCT/US99/20881, U.S. Provisional Application No. 60/155,493, and U.S. Application Serial No. 09/404,133.

17. As shown in part by the representative documents referred to in the preceding paragraphs, during the period prior to September 24, 1998, until September 23, 1999, we were engaged in substantially continuous activity to make enzymatically active human Asp2 protein lacking a transmembrane domain, using materials and methods that we had contemplated in our September 24, 1998, patent application and/or had produced by that September 24, 1998 filing date.

IV. Certification

18. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: November 30, 2001

Michael J. Bienkowski

Michael Jerome Bienkowski, Ph.D.

Exhibit A

Figure 3 Alignment of Prosite Aspartyl protease consensus sequence with active site motifs in Hu_Asp-2

[LIVMFGAC]-[LIVMTADN]-[LIVFSA]-D-[ST]-G-[STAV]-[STAPDENQ]-X-[LIVMFSTNC]-X-[LIVMFGTA]

N-Terminal motif: ILVDTGSSNFAV

[LIVMFGAC]-[LIVMTADN]-[LIVFSA]-D-[ST]-G-[STAV]-[STAPDENQ]-X-[LIVMFSTNC]-X-[LIVMFGTA]

C-Terminal motif: SIVDSGTTNLRL

Figure 4

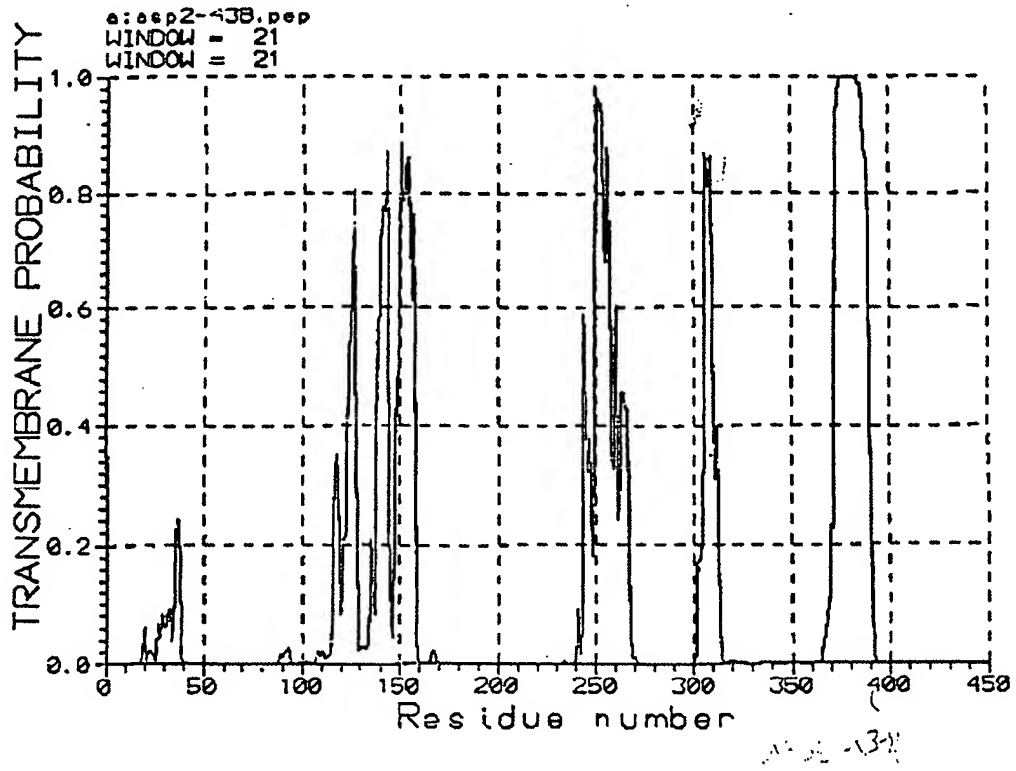


Exhibit B

In a preferred embodiment, the nucleic acid molecules comprise a polynucleotide having a nucleotide sequence selected from the group consisting of residues 21-1290 of SEQ ID NO:1, encoding Hu-Asp1, residues 84-1325 of SEQ ID NO:3, encoding Hu-Asp2(a), and residues 84-1400 of SEQ ID NO:5, encoding Hu-Asp2(b). In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent conditions to a polynucleotide encoding Hu-Asp1, Hu-Asp2(a), Hu-Asp-2(b), or fragments thereof. European patent application EP 0 848 062 discloses a polypeptide referred to as "Asp 1," that bears substantial homology to Hu-Asp1, while international application WO 98/22597 discloses a polypeptide referred to as "Asp 2," that bears substantial homology to Hu-Asp2a.

The present invention also provides vectors comprising the isolated nucleic acid molecules of the invention, host cells into which such vectors have been introduced, and recombinant methods of obtaining a Hu-Asp1, Hu-Asp2(a), or Hu-Asp2(b) polypeptide comprising culturing the above-described host cell and isolating the relevant polypeptide.

In another aspect, the invention provides isolated Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides, as well as fragments thereof. In a preferred embodiment, the Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides have the amino acid sequence given in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6, respectively. Isolated antibodies, both polyclonal and monoclonal, that bind specifically to any of the Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides of the invention are also provided.

The invention also provides a method for the identification of an agent that modulates the activity of any of Hu-Asp-1, Hu-Asp2(a), and Hu-Asp2(b).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Figure 1 shows the nucleotide (SEQ ID NO:1) and predicted amino acid sequence (SEQ ID NO:2) of human Asp1.

Figure 2: Figure 2 shows the nucleotide (SEQ ID NO:3) and predicted amino acid sequence (SEQ ID NO:4) of human Asp2(a).

Figure 3: Figure 3 shows the nucleotide (SEQ ID NO:5) and predicted amino acid sequence (SEQ ID NO:6) of human Asp2(b). The predicted transmembrane domain of Hu-Asp2(b) is enclosed in brackets.

Figure 4: Figure 4 shows the sequence (SEQ ID NO:) of APP695 C-terminus after addition of the di-Lys motif using "patch" PCR.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes a method to scan gene data bases for the simple active site motif characteristic of aspartyl proteases. Eukaryotic aspartyl proteases such as pepsin and renin possess a two domain structure which folds to bring two aspartyl residues into proximity within the active site. These are embedded in the short tripeptide motif DTG, or more rarely, DSG. Most aspartyl proteases occur as proenzyme whose N-terminus must be cleaved for activation. The DTG or DSG active site motif appears at about residue 65-70 in the proenzyme (prorenin, pepsinogen), but at about residue 25-30 in the active enzyme after cleavage of the N-terminal prodomain. The limited length of the active site motif makes it difficult to search collections of short, expressed sequence tags (EST) for novel aspartyl proteases. EST sequences typically average 250 nucleotides or less, and so would encode 80-90 amino acid residues or less. That would be too short a sequence to span the two active site motifs. The preferred method is to scan data bases of hypothetical or assembled protein coding sequences. The present invention describes a computer method to identify candidate aspartyl proteases in protein sequence data bases. The method was used to identify seven candidate aspartyl protease sequences in the Caenorhabditis elegans genome. These sequences were then used to identify by homology search Hu-Asp1 and two alternative splice variants of Hu-Asp2, designated herein as Hu-Asp2(a) and Hu-Asp2(b).

In another embodiment, the invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a polypeptide selected from the group consisting of human aspartyl protease 1 (Hu-Asp1) and two alternative splice variants of human aspartyl protease 2 (Hu-Asp2), designated herein as Hu-Asp2(a) and Hu-Asp2(b). As used herein, all references to "Hu-Asp2" should be understood to refer to both Hu-Asp2(a) and Hu-Asp2(b). Hu-Asp1 is expressed most abundantly inpancreas and prostate tissues, while Hu-Asp2(a) and Hu-Asp2(b) are expressed most abundantly in pancreas and brain tissues, with low levels of expression observed in all other tissues examined except thymus and PBLs. The invention also provides isolated Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides, as well as fragments thereof which exhibit aspartyl protease activity.

The predicted amino acid sequences of Hu-Asp1, Hu-Asp2(a) and Hu-Asp2(b) share significant homology with previously identified mammalian aspartyl proteases such as pepsinogen A, pepsinogen B, cathepsin D, cathepsin E, and renin. P.B.Szecs, *Scand. J. Clin. Lab. Invest.* 52:(Suppl. 210 5-22 (1992)). These enzymes are characterized by the presence of a duplicated DTG/DSG sequence motif. The Hu-Asp1 and HuAsp2

may be similar to or significantly different from a native Hu-Asp polypeptide in molecular weight and glycosylation pattern. Expression of Hu-Asp in bacterial expression systems will provide non-glycosylated Hu-Asp.

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. Hu-Asp polypeptides may be recovered and purified from recombinant cell cultures by well-known methods, including ammonium sulfate or ethanol precipitation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. In a preferred embodiment, high performance liquid chromatography (HPLC) is employed for purification.

The present invention also relates to vectors comprising the polynucleotide molecules of the invention, as well as host cell transformed with such vectors. Any of the polynucleotide molecules of the invention may be joined to a vector, which generally includes a selectable marker and an origin of replication, for propagation in a host. Because the invention also provides Hu-Asp polypeptides expressed from the polynucleotide molecules described above, vectors for the expression of Hu-Asp are preferred. The vectors include DNA encoding any of the Hu-Asp polypeptides described above or below, operably linked to suitable transcriptional or translational regulatory sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, mRNA ribosomal binding sites, and appropriate sequences which control transcription and translation. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the DNA encoding Hu-Asp. Thus, a promoter nucleotide sequence is operably linked to a Hu-Asp DNA sequence if the promoter nucleotide sequence directs the transcription of the Hu-Asp sequence.

Selection of suitable vectors to be used for the cloning of polynucleotide molecules encoding Hu-Asp, or for the expression of Hu-Asp polypeptides, will of course depend upon the host cell in which the vector will be transformed, and, where applicable, the host cell from which the Hu-Asp polypeptide is to be expressed. Suitable host cells for expression of Hu-Asp polypeptides include prokaryotes, yeast, and higher eukaryotic cells, each of which is discussed below.

The Hu-Asp polypeptides to be expressed in such host cells may also be fusion proteins which include regions from heterologous proteins. Such regions may be included to

allow, *e.g.*, secretion, improved stability, or facilitated purification of the polypeptide. For example, a sequence encoding an appropriate signal peptide can be incorporated into expression vectors. A DNA sequence for a signal peptide (secretory leader) may be fused in-frame to the Hu-Asp sequence so that Hu-Asp is translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cell promotes extracellular secretion of the Hu-Asp polypeptide. Preferably, the signal sequence will be cleaved from the Hu-Asp polypeptide upon secretion of Hu-Asp from the cell. Non-limiting examples of signal sequences that can be used in practicing the invention include the yeast I-factor and the honeybee melatin leader in sf9 insect cells.

In a preferred embodiment, the Hu-Asp polypeptide will be a fusion protein which includes a heterologous region used to facilitate purification of the polypeptide. Many of the available peptides used for such a function allow selective binding of the fusion protein to a binding partner. For example, the Hu-Asp polypeptide may be modified to comprise a peptide to form a fusion protein which specifically binds to a binding partner, or peptide tag. Non-limiting examples of such peptide tags include the 6-His tag, thioredoxin tag, hemaglutinin tag, GST tag, and OmpA signal sequence tag. As will be understood by one of skill in the art, the binding partner which recognizes and binds to the peptide may be any molecule or compound including metal ions (*e.g.*, metal affinity columns), antibodies, or fragments thereof, and any protein or peptide which binds the peptide, such as the FLAG tag.

Suitable host cells for expression of Hu-Asp polypeptides include prokaryotes, yeast, and higher eukaryotic cells. Suitable prokaryotic hosts to be used for the expression of Hu-Asp include bacteria of the genera *Escherichia, Bacillus, and Salmonella,* as well as members of the genera *Pseudomonas, Streptomyces,* and *Staphylococcus.* For expression in, *e.g.*, *E. coli*, a Hu-Asp polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in a prokaryotic host. The N-terminal Met may optionally then be cleaved from the expressed Hu-Asp polypeptide.

Expression vectors for use in prokaryotic hosts generally comprise one or more phenotypic selectable marker genes. Such genes generally encode, *e.g.*, a protein that confers antibiotic resistance or that supplies an auxotrophic requirement. A wide variety of such vectors are readily available from commercial sources. Examples include pSPORT vectors, pGEM vectors (Promega), pPROEX vectors (LTI, Bethesda, MD), Bluescript vectors (Stratagene), and pQE vectors (Qiagen).

Hu-Asp may also be expressed in yeast host cells from genera including *Saccharomyces, Pichia,* and *Kluveromyces.* Preferred yeast hosts are *S. cerevisiae* and *P. pastoris.* Yeast vectors will often contain an origin of replication sequence from a 2T yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Vectors replicable in both yeast and *E. coli* (termed shuttle vectors) may also be used. In addition to the above-mentioned features of yeast vectors, a shuttle vector will also include sequences for replication and selection in *E. coli.* Direct secretion of Hu-Asp polypeptides expressed in yeast hosts may be accomplished by the inclusion of nucleotide sequence encoding the yeast I-factor leader sequence at the 5' end of the Hu-Asp-encoding nucleotide sequence.

Insect host cell culture systems may also be used for the expression of Hu-Asp polypeptides. In a preferred embodiment, the Hu-Asp polypeptides of the invention are expressed using a baculovirus expression system (*see* Example 3). Further information regarding the use of baculovirus systems for the expression of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988).

In another preferred embodiment, the Hu-Asp polypeptide is expressed in mammalian host cells. Non-limiting examples of suitable mammalian cell lines include the COS-7 line of monkey kidney cells (Gluzman *et al., Cell* 23:175 (1981)) and Chinese hamster ovary (CHO) cells. Preferably, human embryonic kidney cell line 293 is used for expression of Hu-Asp proteins.

The choice of a suitable expression vector for expression of the Hu-Asp polypeptides of the invention will of course depend upon the specific mammalian host cell to be used, and is within the skill of the ordinary artisan. Examples of suitable expression vectors include pcDNA3 (Invitrogen) and pSVL (Pharmacia Biotech). A preferred vector for expression of Hu-Asp polypeptides is pBK-CMV (Stratagene). Expression vectors for use in mammalian host cells may include transcriptional and translational control sequences derived from viral genomes. Commonly used promoter sequences and enhancer sequences which may be used in the present invention include, but are not limited to, those derived from human cytomegalovirus (CMV), Adenovirus 2, Polyoma virus, and Simian virus 40 (SV40). Methods for the construction of mammalian expression vectors are disclosed, for example, in Okayama and Berg (*Mol. Cell. Biol. 3*:280 (1983)); Cosman *et al.* (*Mol. Immunol.*

23:935 (1986)); Cosman *et al.* (*Nature 312*:768 (1984)); EP-A-0367566; and WO 91/18982.

The polypeptides of the present invention may also be used to raise polyclonal and monoclonal antibodies, which are useful in diagnostic assays for detecting Hu-Asp polypeptide expression. Such antibodies may be prepared by conventional techniques. See, for example, *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1988); *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet *et al.* (eds.), Plenum Press, New York (1980).

The Hu-Asp nucleic acid molecules of the present invention are also valuable for chromosome identification, as they can hybridize with a specific location on a human chromosome. Hu-Asp1 has been localized to chromosome 21, while Hu-Asp2 has been localized to chromosome 11. There is a current need for identifying particular sites on the chromosome, as few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. The relationship between genes and diseases that have been mapped to the same chromosomal region can then be identified through linkage analysis, wherein the coinheritance of physically adjacent genes is determined. Whether a gene appearing to be related to a particular disease is in fact the cause of the disease can then be determined by comparing the nucleic acid sequence between affected and unaffected individuals.

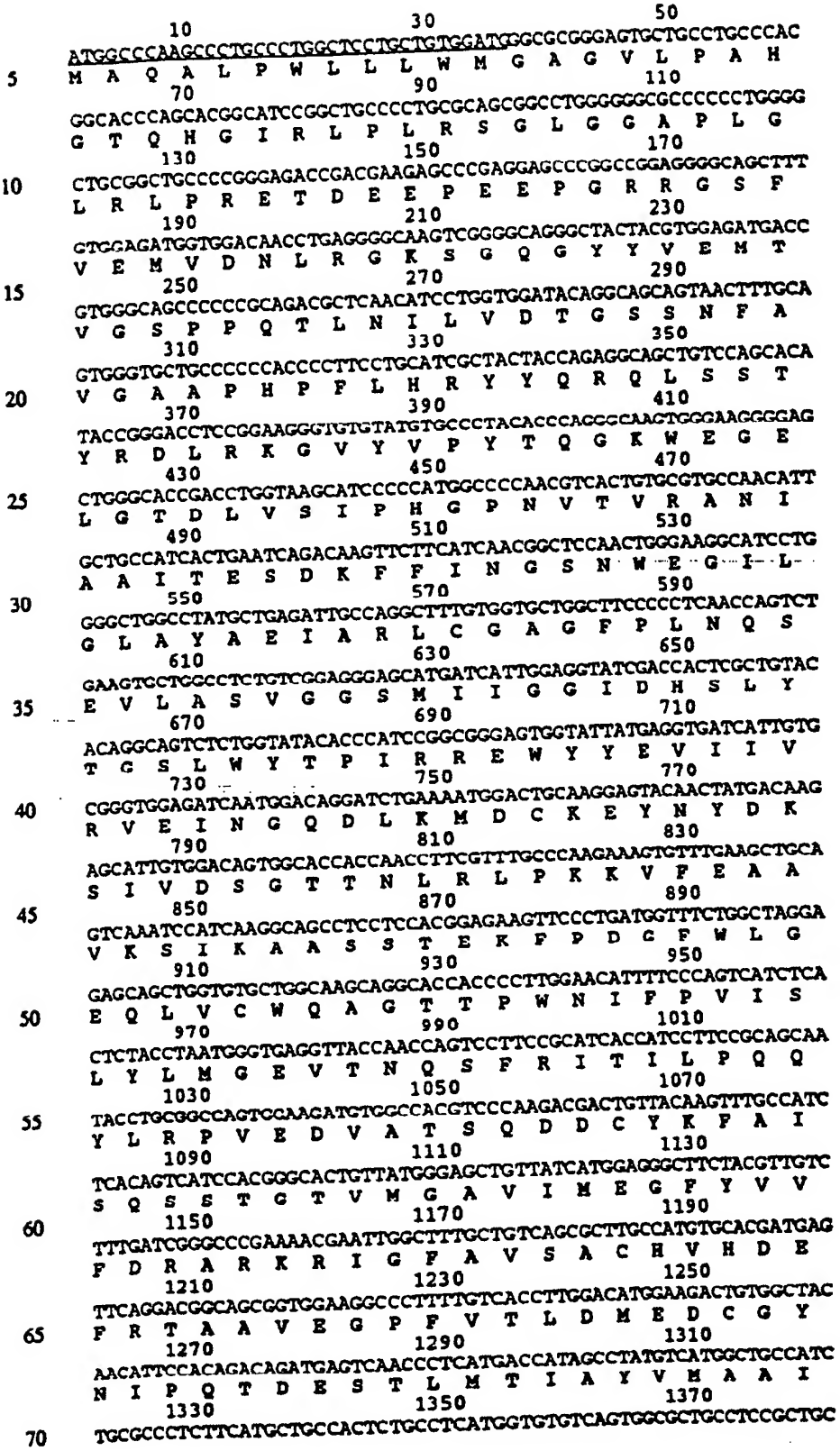
In another embodiment, the invention relates to a method for the identification of an agent that increases the activity of a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), the method comprising

(a) determining the activity of said Hu-Asp polypeptide in the presence of a test agent and in the absence of a test agent; and

(b) comparing the activity of said Hu-Asp polypeptide determined in the presence of said test agent to the activity of said Hu-Asp polypeptide determined in the absence of said test agent;

whereby a higher level of activity in the presence of said test agent than in the absence of said test agent indicates that said test agent has increased the activity of said Hu-Asp polypeptide.

FIGURE 2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6177

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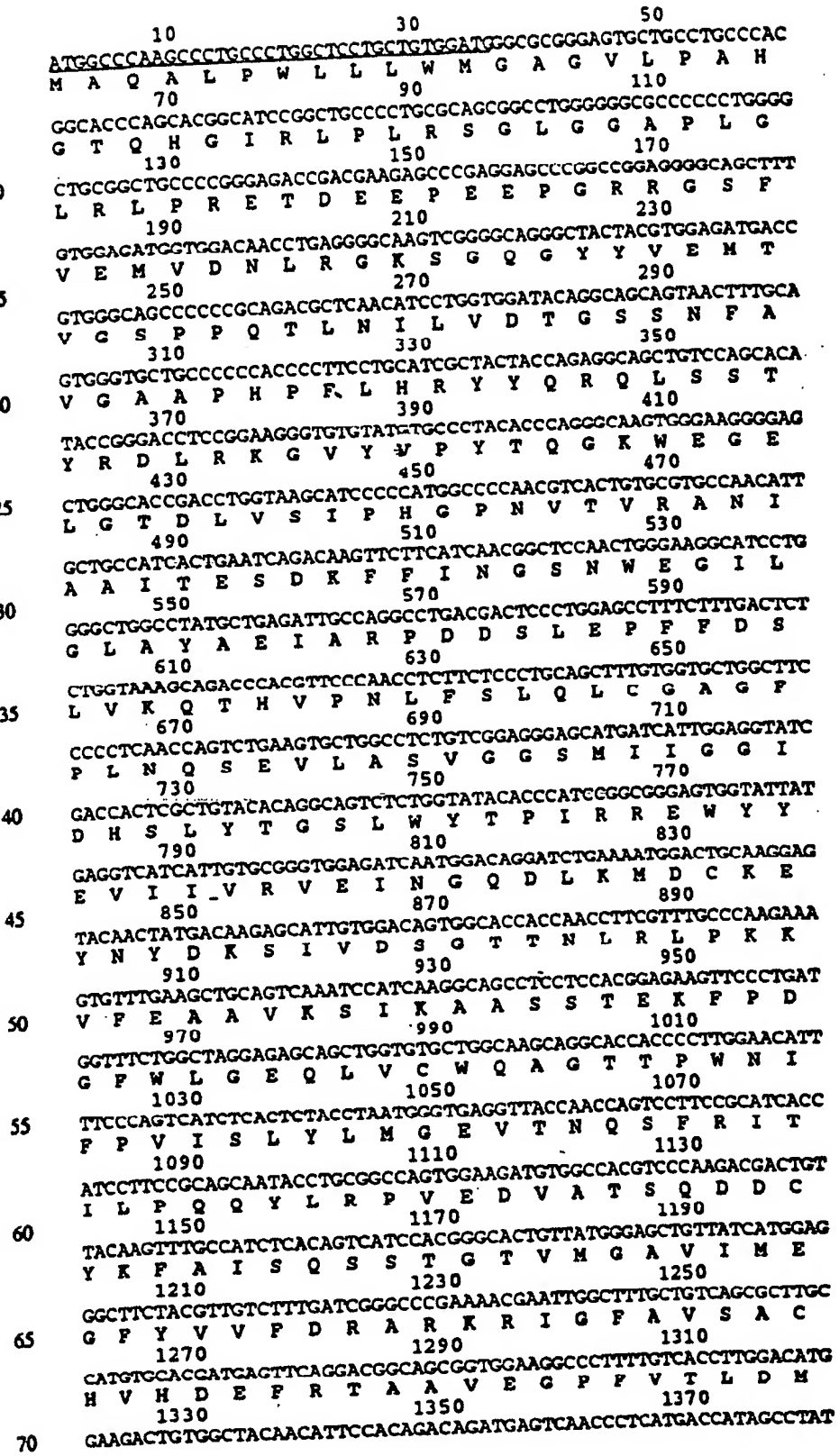
FIGURE 3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                1870                    1890                    1910
GTACTGGCATCACACGCAGGTTACCTTGGCGTGTGTCCCTGTGGTACCCTGGCAGAGAAG
                1930                    1950                    1970
AGACCAAGCTTGTTTCCCTGCTGGCCAAAGTCAGTAGCAGAGGATGCACAGTTTGCTATT
                1990                    2010                    2030
TGCTTTAGAGACAGGGACTGTATAAACAAGCCTAACATTGGTGCAAAGATTGCCTCTTGA
                2050                    2070
ATTAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Exhibit C

coding sequence of Hu_Asp-1 has been prepared and the predicted amino acid sequence, aligned with both the short and long forms of Hu_Asp-2, is attached. This splice variant of Hu_Asp-1 encodes a 521 amino acid polypeptide including a 27 residue signal peptide so the pro-form of the enzyme contains 76 amino acid residues upstream of the first active site motif. This upstream sequence also contains a third DSG motif. Alignment of the sequence surrounding this upstream DSG with the ProSite motif for aspartyl proteases revealed a poor match while the other two DTG/DSG motifs showed a good match. Alignment, with Hu_Asp-2 sequences using the Clustal W algorithm highlights two major differences between Hu_Asp-1 and Hu_Asp-2; the NH_2 terminal extension in Hu_Asp-1 is much longer and that Hu_Asp-1 appears to be more like the long form of Hu_Asp-2. The longest stretches of amino acid identity align with the two aspartyl protease active site motifs although other areas of conservation are also scored.

Finally, the Hu_Asp-1 gene was localized to human Chromosome 21 by hybridization to a Southern blot containing a series of mouse/human or hamster/human somatic cell hybrids (attached).

Hu_Asp-2, Mary provided an inventory of the expression constructs for Hu_Asp-2 (attached). The entire ORF of both the short (438) and long forms (269) of Hu_Asp-2 have be engineered into the mammalian cell expression vector pBK-CMV. Also, both the short

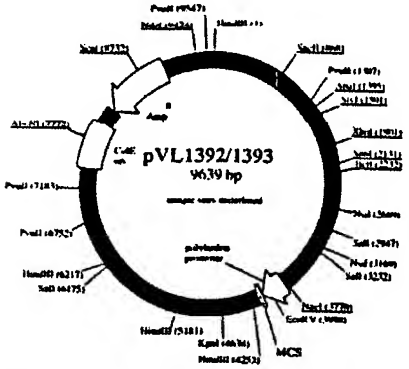
and long forms, with the COOH-terminal transmembrane domain deleted, have been prepared as NH_2 terminal 6His-fusions in the *E. coli* expression vector pQE30. Finally, the entire ORF from the short form of Hu_Asp-2 has been cloned downstream of the ecdysone-inducible promoter in the vector pIND and in a polycistronic fusion with GFP (pIRESGFP) for mammalian cell expression studies.

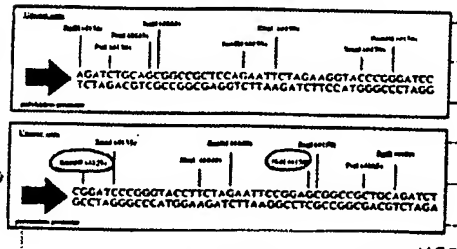
Hu_Asp-3 and Hu_Asp-4— Queries of the LifeSeq Assembled database with the sequences of either Hu_Asp-1 or Hu_Asp-2 identified (1) gene bins with exact matches to the query sequences, (2) gene bins matching the 5 known human aspartyl proteases [pepsinogen A, pepsinogen C, cathepsin D, cathepsin E and renin], and (3) three gene bins with significant homology [242842, 242824, 39511], in descending order of significance. Translation of the longest assembled templates contained within these gene bins revealed that they each encoded polypeptides containing the duplicated active site motif that is the hallmark of mammalian aspartyl proteases. Alignment of the predicted amino acid sequences for templates 451054.3 and 451034.4 showed that they were very similar with approximately 90% sequence identity at the amino acid level (attached). Template 126360 was most related to 451054.3 and 451034.4, with approximately 70% shared identity. Consistent with the nomenclature initiated previously, the genes represented by Incyte templates 451054.3, 451034.4, and 126360 are referred to as Hu_Asp-3, Hu_Asp-4a and Hu_Asp-5, respectively. Template 451034.2 appeared to be a splice variant of 450134.4 with a 25 amino acid (75 bp) insertion near the CO_2H-terminus (data not shown). The cDNAs that defined the 5'-most sequence of each of these templates were identified, obtained for sequence analysis and determination of the tissue distribution of expression of transcripts derived from these genes. The Hu_Asp-3 probe visualized a single 1.6 kb transcript that showed a limited expression pattern that was expressed at the highest levels in lung, immunological tissues (spleen, thymus and PBLs), and kidney (attached). No expression of Hu_Asp-3 transcripts was detected in whole brain while a weak signal was observed in several brain regions including the medulla, spinal cord and putamen (attached). These results were consistent with the expression pattern determined by EST sequencing in LifeSeq Assembled (39 ESTs) which indicated highest expression in the hematopoietic/immune category (41%) and the nervous category being the second highest (16%). The Hu_Asp-4 p visualized a similar pattern of transcript size and abundance except that the signal was mos in lung tissue. No transcripts were detected in either whole brain or selected brain regions u conditions used in these experiments. A survey of expression using LifeSeq Assembled (1 indicated that 93% of the ESTs that comprise the Hu_Asp-4 template were derived from r

Exhibit D

Asp2 → Baculovirus Expression

Engineer the pre-pro form of Asp2 ± TM for expression in Baculovirus using the Vector pVL1393





BAM Kozak

GGATCC GCC ACC ATG GCC ...

G S S T M A Q A L ...

PCR: 100ng pcDNA3.1 hygro Asp2 ® 1µl
8µl dNTPs
5 Pwo buf
1.5 Asp2BAM
(x2) or Asp2not-tm — 1.5 Asp2-not
1 Pwo I
32 H_2O
15 cycles

Extract, ppt. Digest w/ 83µl H_2O / 10µl 10x ✱3 / 4µl Bam / 3µl Not — 37°C o/n

asp2Bam CGC TTT GGA TCC GCC ACC ATG GCC CAA GCC CTG CCC TGG
BAM S T M A Q A L P W

asp2not-tm GCG TTT GCGGCCGC CTA TCA CTC ATC TGT CTG TGG AAT GTT G
NOT * S E D T Q = reverse complement

asp2not CGC TTT GCGGCCGC TCA CTT CAG CAG GGA GAT GTC ATC
NOT * K L L S I = reverse complement

Run 1% prep gel

Geneclean fragm.

Set up ligation o/N

①
Asp2/BAM-Not (100ng) = 1µl
pVL1393 (160ng) 4µl
8µl

②
Asp2 -tm / BAM Not 130ng = 2µl
pVL1393 160ng = 4µl

14°C o/N

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TF DH5α w/ 2μl Plate on LB+Amp

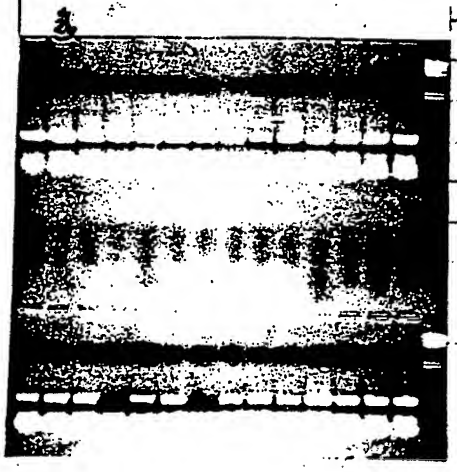
Pick CFUs #[illegible] for long form — tm had no CFUs (#1-8)
PCR w/ Asp2-1 → Asp2-2
See p. 114 — looks good! (#7)
Plate total — tm tf

No — tm ff's ∴ ck frag. o/n @ gel — lighter than expect — Set up new ligation.

— tm ligation: 2μl pVL1393 (80ng)
6μl Asp2 — tm (~55ng)
1μl 10x
1μl ligase
} 14°C o/n

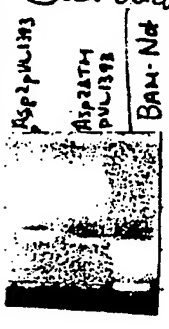
TF DH5α w/ 2μl plate on LB-Amp
Pick 28 CFUs & PCR w/ Asp2-1 & Asp2-4 (427bp)
Asp2 - tm pVL1393 Big PCR
Asp2-1 — 2-4

Pick #2 for Cs prep - MJB



Harvested Cs preps - lots of debris in tubes
Extract, dialyze etc
Conc by OD: Asp2 pVL1393 = 1.37 μg/μl
Asp2ΔTM pVL1393 = 0.93 μg/μl

Test digest w/ BAM+Not @37°C o/n



Given to D. Fisher for Baculo expression & Roger for Seq. confirmation

Seq of ± TM constructs is correct (1 nt change that is silent)

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SW

Exhibit E

Generation of 6-His tagged Asp2 Baculo Constructs

- We have previously prepared Asp2 ± TM (full length-signal, pro et) in the baculo expression vector pVL1393.

- We have recieved the first Clones for Asp1+TM & Asp2+TM. Asp1 has been analyzed by PAGE & Western. There is a ton of protein being produced & is associated with the Sf9 cells. At this stage we can't say if the signal sequence is being clipped, if it's membrane associated or free in the cytosol.

- Anticipating difficulty in the purification of a non-secreted, non-tagged protein I will design primers that incorporate a C-term 6 His. The primers will be as pag #105 Notebook 31942 w/ 6 His added.

Asp2not—tmHis : cgc TTT gcggccgc (NOT I) CTA (*) -(ATg)x6 (6 His) - TgA (S) - CTC (E) ATC - TgT - CTg - Tgg - AAT - gTT - g

Asp2not His : cgc TTT gcggccgc (NOT) - TCA (*) - (ATg)x6 - CTT (K) - CAg (L) - CAg (L) - ggA (S) - gAT (I) - gTC - ATC

ASP2NOT-TMHIS	27629-001	60	DST	0.30	12515.8	61.6	32.8	34.6	807.8	43.6	Moderate	No	CGCTTTGCGGCCGCCTAATGATGATGATGATGATGTGACTCATCTGTCTGTGGAATGTTG
ASP2NOTHIS	27629-002	56	DST	0.30	17271.2	82.8	32.4	22.8	714.5	41.4	Moderate	No	CGCTTTGCGGCCGCTCAATGATGATGATGATGATGCTTCAGCAGGGAGATGTCATC

PCR 100ng 1µl of Asp2 pcDNA3.1hygro+ ®
32µl H_2O
8µl dNTP
5µl 10x buffer
1.5µl Asp2 Bam
1.5µl Asp2not—tmHis or Asp2not His
1µl Pwo I

} x 2 } 15 cycles 9700

Extract & ppt rxns. Resuspend ± tm in 4µl H_2O, 5µl 10X #3, 2µl Bam, 2µl Not } 37°C o/n

Read and understood by [signature] [signature]

Run 1% agarose prep gel:

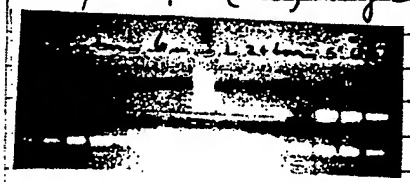
Cut out fragments & gel recover

Conc by OD =
Asp2-tm His = 80ng/µl
Asp2+tm His = 60ng/µl

Ligations: 120ng pVL1393/Not Bam = 3µl
Asp2 insert 2µl
10x buffer 1
T4 ligase 1
H_2O 3
16°C o/N

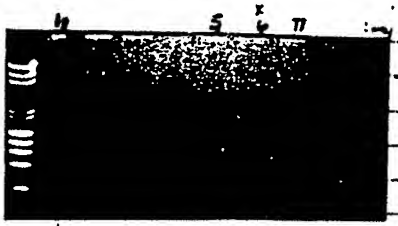
TF DH5 w/ 2µl. Plate 200µl on LB+Amp+Inc @ 37°C o/N

Good # of CFUs over background - Pick 7 CFUs / Construct & PCR w/ Asp2 (2:5) to yield @ ~ 440bp frag)



Nice Neg Control for the —tm's — the primers (Asp2-2) is in the TM — take a guess & pick # to for each Construct Inoc 1 liter LB+Amp=Kan @ 37°C o/n

Asp2 — tm His
PCR'd: 2-70-Bam & 2-70-Sph



: Work up std alkaline lysis preps. Band on Cs = also re PCR — tm bugs w/ new set of primers (2-70-Bam & 2-70-Sph) There are weak bands in 1, 5, 7 — I don't see anything in # 6 but I'm hoping that is due to not having much to amplify in the PCR after using most of the 50µl for the inoculation

Harvest nice plasmid bands, Extract & dialyze / H_2O o/n

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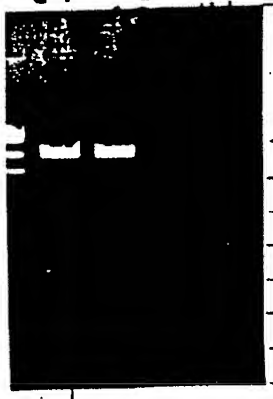
Ppt & Resuspend in 1 ml H_2O

Asp2 + tm His pVL1393 = 1.9 mg/ml
Asp2 − tm His pVL1393 = 2.3 mg/ml

Test digest 1 µl w/ 4 µl Bam + 4 µl Not in 100 µl 1X #3 for ~4 hrs @ 37°C



Look good. Submit to DSC

+TM = A (12r, 13f, 14f, 15r, 16f, 29f, 30r)
−tm = B (")

Note to BEVS to begin baculo expression
Samples given to Ma Li to transfer to Merrill Babcock

Rec'd seq from Roger @ /DSC930-934

The Seq's are correct.

Bam-Not sites intact
6xHis fused on C-terminal followed by STOP
Alignment w/ 2R Seq - 100% identity except ambiguity that has always existed between 2R & SKB — Silent w/ no AA change

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Exhibit F

Pro Asp2 pQE70 Analysis

See 31942 p. 145-148 for construction, etc

4 CFUs from the Asp2pQE70 mp #6 / M15(pREP) transformation were picked & grown & induced w/ 1mM IPTG.
The induction was not obvious.

Next actions: Digest mp #6 looked ok : Send up for sequencing
Rerun more Sample To/T4 on a 10 well gel.

Reran 10µl of To/T4 samples after mechanically shearing w/ a 22g needle, followed by reboiling 5'. The induced time points pinched & smeared while the To's looked ok.
Induction of a single band isn't obvious —
The #6 mp DNA was submitted for Seq w/
12r, 13f, 14f, 15r, 16f, pQE30r Called Pro70
— pQE70 forward (pQE promotor) primer was ordered.

Obtained preliminary Seq from Roger. There are 2 bp deleted & a substitution relative to the correct Seq.

Roger "found" the 2bp deletion — but the 1st substitution which changes N → T can't be read from either strand: resubmit w/ primer #16f & give Roger 2x11µl DNA so he can add some of his own Asp2 primers

Jerry said the samples were dropped — resubmitted this (A.M.)

Even if there is an amino acid substitution, it should be making & inducing a protein since all is in frame w/out STOPS

Roger left a message saying "It's an A" which makes the Seq correct

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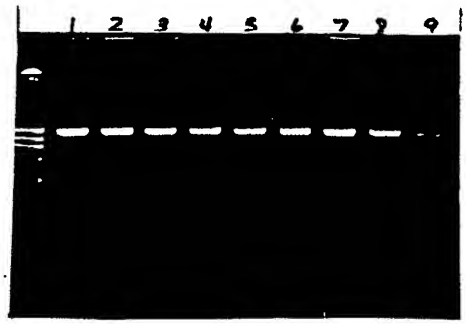
Pick 9 CFU from Asp2 #6 pQE70[M15pREP] & PCR to verify inserts w/ 2-70-Spn & 2-70-Bam for 35 cycles:

Asp2pQE70[M15pREP] 2-70-Spn & 2-70-Bam



All are positive as would be expected since the M15's were tf w/ supercoiled mp #6 DNA.

Inoc 2x5ml LB + 100μg/ml Amp + 25μg/ml Kan w/ #1-4 - Grow 2½ hrs - freeze 1x 5ml aliquot

IPTG induce the other. (1mM) for 3 hrs - freeze cultures

Thaw cultures ck OD of 1ml (*: IPTG induced)

1	0.464	pellet 1ml ~ 0.500	Resuspend in 50μl E+/SDS Δ100°C 5' Add 15μl H_2O 25μl 4X NuPAGE S.B. 10μl reducing agent
2	0.554		
3	0.475		
4	0.428		
*1	0.823	pellet 0.5ml ~ 0.500	
*2	0.895		
*3	0.928		
*4	0.921		

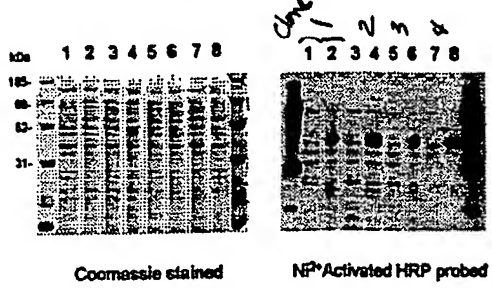
Run 2 NuPAGE 10% MES gels w/ 10μl of each sample marker 1 1°, 2 2.2° etc double marker.

Stain 1 gel in colloidal blue
Western blot 1 gel - probe w/ Pierce INDIA His-Probe Super Signal

Stain shows no obvious induction, but the His-probe clearly picks up an induced band in all 4 clones.

A very faint band can be seen that corresponds

ProAsp2-TMpQE70 Expression in E.coli



Coomassie stained Ni²⁺Activated HRP probed

Inoc 400ml LB + Amp + Kan w/ #1. Grow @ 37°C o/N

Grow 4 liters LB + Amp + Kan Inoc each w/ 100ml o/N culture. Grow 2½ hrs Induce w/ IPTG to 1mM for 3 hrs

Spin down bugs & transfer to M. Fairbanks

Mike says he sees the His signal in the Soluble fraction — but at very low levels
Will try a time course to try & boost expression

In an effort to boost expression - Try a time course & Switch to clone #2

5ml LB + 25µg/ml Kan + 100µg/ml Amp

Inoc 8 x 5ml LB + 200µg/ml AMP + 25µg/ml Kan w/ 50µl o/N
Grow @ 37°C 2½ hrs

Induce 4 x 5ml w/ 1mM IPTG
4 x 5ml w/ 2mM IPTG

Collect time points @ 1hr, 2hr, 4hr, o/N → Store on ice @ 4°C

Ck OD A_{600} of each culture. Pellet 1 OD of each & give to M. Fairbanks for analysis

M. Fairbanks reports no expression.

Now Hendrickson reports that Jordan Jarap Colleagues

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have successfully expressed active Asp 2 in E coli
Suddenly the focus has shifted back to E coli expression

Trying to work up the ProΔTM pQE70 Construct we have in hand & optimize expression:

TF ProAsp2pQE70 #6 mp DNA into XL-1 Blue MRF' Kan
DH5α F'IQ

Plate on LB+Amp (100 μg/ml) + Kan (25 μg/ml) plates w/ Inc @ 32°C o/N

Prepare:	LB-LS	LB-HS	2XYT	SuperBroth
Tryptone	10	10	16	35
yeast Ext	5	5	10	20
NaCl	5	10	5	5

Pick 2 cells from each transformation → 50 μl H2O
Aliquot 10 μl to 10 ml of each broth w/ Inc @ 37°C w/ Shaking After 8 hrs @ 37°C ck ODs

GAM

Bug	Clone	broth	OD/ml	cells/ml induced
XL1 Blue	1	LB-LS	0.325	1.16
"	1	LB-HS	0.511	1.75 remove 0.5 ea worth of
"	2	LB-LS	0.274	1.73 pellet in 2059 tube.
"	2	LB-HS	0.391	1.74 Store @ 4°C o/N
DH5α	1	LB-LS	0.345	1.67
"	1	HS	0.391	1.69 Induce 5 ml w/
"	2	LS	0.132	1.68 50 μl 100 mM IPTG
"	2	HS	0.103	1.70 (1 mM) @ 30°C
XL1 Blue	1	2XYT	0.543	1.92
"	2	2XYT	0.430	1.92 o/N (14½ hrs)
DH5α	1	2XYT	0.483	1.89
"	2	2XYT	0.147	1.9 Remove 0.5 cc of
XL1 Blue	1	Super	0.617	1.99 Induced & pellet
"	2	"	0.416	1.97
DH5α	1	"	0.413	1.99
"	2	"	0.116	1.94

See D-69

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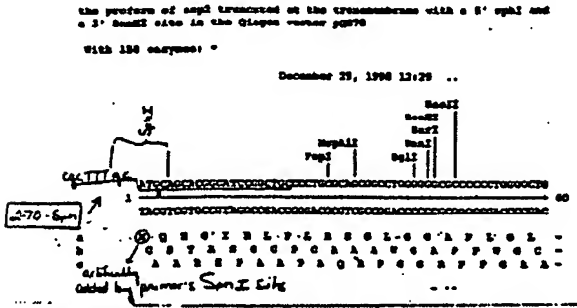
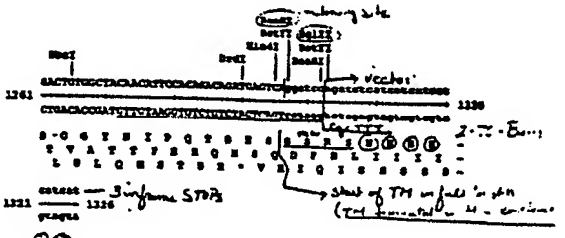
Ecoli Expression: Asp2 Pro Form

Ordered the oligos to allow expression of the Pro form minus the TM in E. coli using the Qiagen vector pQE70

The 5' oligo will incorporate an ATG embedded in an Spn I site, then start with the amino acid sequence QHGIRL...

The 3' oligo will add a Bam HI site immediately 3' to the last amino acid 5' to the TM. The 6 His tag will be incorporated on the C-terminus by the vector





Oligo Name	Oligo #	LEN	Pur	Scale	MW	Tm	µg/OD	OD	µg	nmol	2ndary	Dimer	Sequence (5'-3')
GEL STANDARD	18mer, ~6mer...												
2-70-SPH	25824-001	30	DST	0.20	9131.0	89.2	34.6	48.8	1681.1	184.1	Strong	No	CGCTTTGCATGCAGCACGGCATCCGGCTGC
2-70-BAM	25824-002	37	DST	0.20	11332.4	81.0	33.6	49.3	1658.0	146.3	Weak	No	CGCTTTGGATCCTGACTCATCTGTCTGTGCAATGTTG

PCR Asp2 pcDNA3.1hygro & Asp2S pcDNA3.1hygro

1µl - 100ng template
5µl 10x Pwo buffer
1.5µl Spn primer
1.5µl BamHI primer
8µl dNTPs
32µl H_2O
1µl Pwo I

2 rxns — 2 — 15 cycles — 2S

Ppt, pellet resuspend in 82µl H_2O
10µl 10x #2
4µl Bam
4µl Spn

37°C O/N

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Also 3µg pQE70 in 40µl

SW

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Run 1% prep gel - See p. 149

Denuclean pQE70/Spn-Bam
Asp2/Spn-Bam
> Resuspend in 50µl

Lose Asp2S - the seq has 2 nt dilutions per J. Slighton

I accidently loaded these fragments back on a gel - cut out & hold @ 4°C

Denuclean frags
ck conc of frags by OD
Asp2/Spn-Bam = 35ng/µl
pQE70/Spn-Bam = 15ng/µl

Ligation:

	③	④	
70ng Asp2 = 2µl		—	
90ng pQE70 = 6		6	16°C o/N
10x buffer	1	1	
ligase	1	1	
H_2O	—	2	

Note
If DH5α w/ 2µl because of the higher tf efficiency - then
Retransform later into (M15pREPs)
Plate 200µl on LB-Amp Inc @ r.t. for the weekend

Only 6 CFU - Pick & PCR w/ Spn-Bam PCR primers.

No inserts!
Start Again w/ the PCR 100ng Asp2pcDNA3.1 = 1µl
dNTPs 8
10x buffer 5 x2
2-70-Spn 1.5
2-70-Bam 1.5 15cycles
Pwo | Date
H_2O 32

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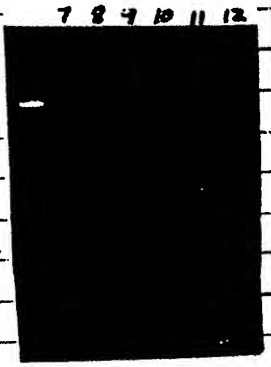
SMK

Pro Asp2pQE70
2-70-Spn · 2-70-Bam
7 8 9 10 11 12



Extract & ppt rxns. Resuspend in 41µl H_2O, 5µl 10X #2, 2µl Spn, 2µl Bam
Also Digest more pQE70 : 5µg = 10µl

10X #2	5
Bam	2
Spn	2
H_2O	31

incu @ 37°C o/N

Del purify w/ geneclean

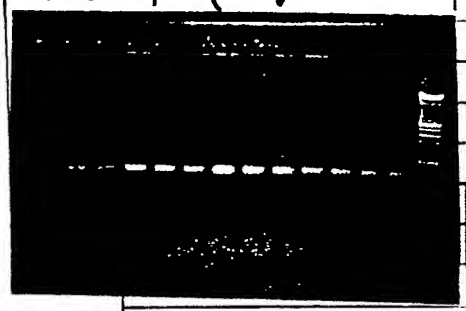
Conc by OD - pQE70/Spn-Bam = 40ng/µl
proAsp2/ " = 20ng/µl

Ligations : 2µl pQE70 - 80ng
1µl 10X
1µl ligase
6µl proAsp2

TF DH5 w/ 2µl. Plate 200µl on LB+KMP

High background. Pick 14 CFUs & PCR w/ Asp2 (2-5) to produce @ ~440bp product.

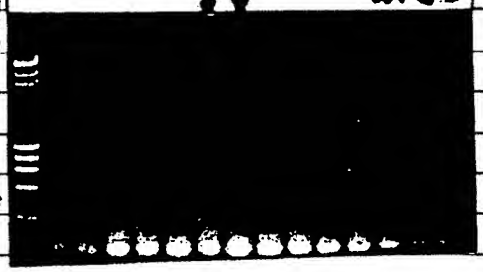
PCR: Asp2 (2-5)



Nice Neg Control ! the primer pair I chose for PCR included the TM ∴ these CFUs may not be neg. Repeat using 2-70-Spn & 2-70-Bam

PCR · 2-70-Bam
2-70-Sp

ProAsp2 pQE70



Faint bands in #6 & #7
incu 5ml o/n w/ #6, #7 for mini preps

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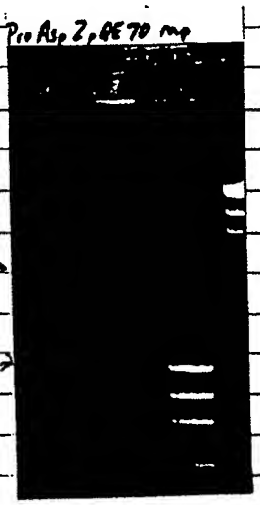
SHW

Work up std. alkaline lysis mini prep from 1½ mls. Resuspend in 100µl H_2O

Test digest 5µl w/
6µl 10x #2
2µl SpnI
2µl BamHI
45µl H_2O
} 2 hrs @ 37°C



about light
#6 looks ok. Tf M15(pREP) 200µl w/ ~0.5µl of #6 & #7. Plate 50µl on LB+Amp+25µg/ml Kan. Incu @ 37°C o/N →

Pick 4 CFU & inoc 5ml LB+Amp+Kan o/n
also inoc 10ml LB+Amp+Kan w/ 500µl of each o/n
Grown ~2 hrs to O.D 0.5–0.7

To		
1	0.555	pellet 1 OD - Store @ -20°C
2	0.57	
3	0.741	
4	0.653	

Add IPTG to 1mM & Continue Growth @ 37°C. Measure OD @ 2hrs

T_2		pellet 1 OD Store -20°C	T_4		pellet 1 OD & Store @ -20°C
1	1.119		1	1.386	
2	1.144		2	1.434	
3	1.267		3	1.464	
4	1.177		4	1.275	

Resuspend 100 equiv's in 65µl E+/SDS. Δ 100°C 2". Add 25µl Novex 4x S.B & 10µl reducing agent Δ 70°C 10'
Run a 15 well NuPAGE gradient gel – loading the 5µl was very difficult due to stringy viscosity (DNA?) Stain gel in Colloidal blue – nothing jumps right out & the loads seem light.

For further analysis See 32587 p. 31

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SW

Exhibit G

Transfection of Sf9 cells with Asp2 Δ TM
(from Bienkoski's lab)

(1) Use 2×10^6 Sf9 cells for transfection.

(2) Add 0.5 µg of virus DNA and 2 µg of transfer DNA.

(3) Incubate at 27°C for 4 hr.

(4) Add 4 ml of TNM medium and keep at 27°C for 5 more days.

. I did co-transfection today, and the cells were incubate at 27°C for 5 more days.

. Transfection stock was harvested and labeled this morning and it was stored at 4°C.

. I did plaque assay today with 6 dilutions of the transfection stock. The plates were kept at 27°C for 6 to 10 more days.

. Five clones were picked up and 5 ml of TNM medium was added into each clone. They will cultune for 3 days at 27°C.

. The 1st Amp stock was harvested and labeled this morning. It was stored at 4°C.

. I did 2nd Amp today and it were kept at 27°C

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for 64 hr.

Harvested all 5 clones this morning. They were labeled as 2nd Amp Stocks and stored at 4°C. Mike came over to picked up both pellets & sups of all 5 clones for assay.

Mike sent me a note send, there is no expression in all 5 clones. He asked to repeat the small infection in the serum free medium, he will assay them again.

I asked Jerome to repeat the small infection of all 5 clones in the serum free medium for Mike.

Jerome told me that after small infection in the serum free medium, Mike chose clone #1 for making a 100 ml of prep.

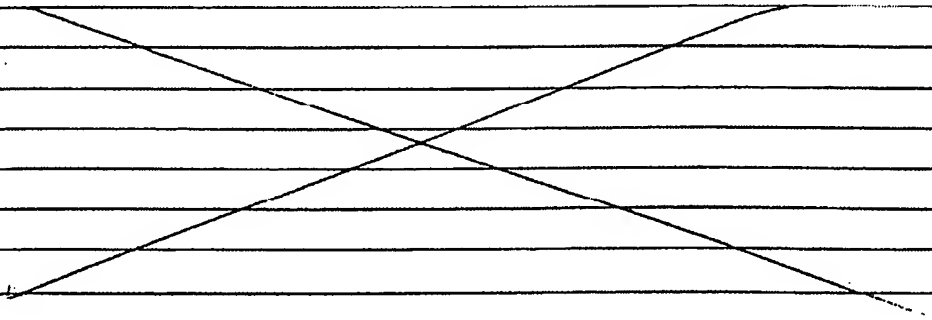

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Exhibit H

Expression Analysis of BVES-Hu Asp2L ΔTM

Purpose: I previously analyzed the analytical scale sf9 cell infections w/ pVL1393/Hu Asp2L ΔTM and concluded that (1) Clone #1 was best for scale-up & (2) it appear that little if any of the target protein is secreted into the medium.

Experimentals:

① More detailed analysis of the Conditioned Medium

Inspection of the WB's of the concentrated conditioned medium did reveal a weakly stained band @ ~ 65kDa that did not appear in the sf9 control (although this may have been due to variable serum content). Since the large albumin band might obscure any secreted Asp2L ΔTM, I decided to fractionate and the Asp2L ΔTM content of the medium may be too low to readily detect by WB analysis, I decided to fractionate the CM.

The protein content of the conditioned medium was quantified using the Biorad method and the results are summarized below.

CM Sample	A^{595} (25µl)	mg/µl	Total (mg)
sf9 control	0.135	0.18	8.1
AcNPV-CDK5-3	0.132	0.17	7.3
Hu Asp2L ΔTM	0.119	0.16	7.2

2 ml aliquots of AcNPV-CDK5-3 and Hu Asp2L ΔTM conditioned medium were dialyzed against 4L (2x) 25mM NaOAc (4.5) o/N @ 4°C. This resulted in some ppt so the solutions were clarified by centrifugation (3000rpm / 15') & the protein assay repeated.

Sample	Suspension		Super
AcNPV-CDK5-3	0.119 / 0.16 mg/µl	3.5 mg total	0.041 / 0.06 mg/ml / 1.3 mg
Hu Asp2L ΔTM	0.092 / 0.13 mg/µl	2.8 mg total	0.039 / 0.06 mg/ml / 1.3 mg

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Based on the protein assays, the recovery following dialysis is as follows:

AcNPT-CDK5-3 1.3mg/3.5mg ×100 = 37%

Hu-Asp2LOTM 1.3mg/3.2mg ×100 = 40%

The clarified supernatants were chromatographed on a MonoS column equilibrated in 25mM NaOAc (4.5) as follows

FR = 1.0ml/min
Sample Load ≈ 22mls
Elution 0 → 100% B, 50' where A = 25mM NaOAc (4.5)
B = " / 1.0M NaCl
The elution profile was monitored @ 280nm (0.05AUFS) & 1.0ml fractions were collected for further analysis.

16.25µl samples were taken for NuPAGE gel analysis as usual;

→ 1X loading buffer + DTT + sample $\xrightarrow[10']{70°C}$ $\xrightarrow{10X\ load}$ 4-12% gradient gels (15well)

→ 1X MES R.B. / ET, 90' @ 35V

→ WB – 1/1000 dil. of 191 TB #4
– 1/2500 dil. of G α R (AP)
– NBT/BCIP

A second gel/WB was run (based on the first gels) to reanalyze the following samples on a 10well/10% gel

1. CHO Asp2L #5 (20)	5. MonoS #12	9. CM (cdk5) control pH 4.5)
2 CM-BVES Asp2LOTM	6. " #14	
3 " " " pH 4.5	7. " #16	
4 MonoS #10	8 CM (cdk5) int	

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Results:

Aliquots of conditioned medium obtained from sf9 cell infections w/ Baculovirus containing the Asp2LΔTM or a control gene (C2K5-3) were analyzed for the presence of Asp2ΔTM protein, before & after chromatography on Mono S & Dialysis of the conditioned medium against NaOAc buffer pH 4.5 led to protein ppt of ~ 2/3 of the original protein. The supernatant was fractionated by Mono S chromatography & the elution profiles monitored by $A^{280\,nm}$ absorbance & WB analysis. Both CM samples showed similar behavior, summarized below

* ~ 2/3 of starting protein ppt upon dialysis
* large A^{280} w/ unbound fraction of Mono S
* similar A^{280} fingerprints during the gradient elution.

To determine which fractions contained Asp2, aliquots were analyzed by WB analysis & the results are summarized below:

* Conditioned Medium ~45kDa immunoreactive band
* Dialyzed CM (Super) ~43kD " "
* PPT from Dialysis — blank

* Mono S column fractions — maybe immunoreactivity in #12

Band (immunoreactive) size decreased following dialysis — ? Activation ??

2ND Analysis

* BVES-CM Asp2LΔTM → immunoreactive band @ 52kDa that is not in the control
* BVES-CM Asp2LΔTM/pH 4.5 → immunoreactive band @ ~ 50kDa, but much lighter than before (not in control)
* faint immunoreactive band in #12/14

* Seems like Asp2LΔTM is going away w/ time @ loss in S/N
* Lack of conc in col

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Exhibit I

Continued Analysis of BVES-Hu Asp2LΔTM Conditioned Medium

Purpose: To determine if purification of the proforms by anion exchange chromatography followed by activation at low pH would be a sensible strategy.

Experimental

The starting material for this work was described on pp 110-112 (both Hu Asp2LΔTM and cdk5 control medium). Twenty mL aliquots of each CM sample were dialyzed against 4 L of 25mM Tris-HCl (pH 8.0) O/N @ 4°C. (A = slight amount of ppt was noted following dialysis so the protein content of the dialysate was quantified before and after centrifugation (8000 rpm, 15'))

Sample	A^{280nm}/5µl	µg/µl	total µg	Δ
BVES-Asp2LΔTM CM-dialysate	0.136	0.18	4,050	
" " - Super	0.121	0.16	3,600	450 µg
BVES-cdk5 CM-dialysate	0.122	0.16	4,000	
" " - Super	0.118	0.15	3750	250 µg

The clarified supernatant obtained following dialysis/centrifugation was chromatographed on a MonoQ column under the following conditions:

- load ~ 22 mL @ 1.0 mL/min w/ 25mM TRIS-HCl (8.0)
- Wash w/ 25mM TRIS-HCl (8.0) until A^{280} reduced (to ~ 30%, never reached 0)
- Elute w/ a 30' gradient from 0 -> 1.0M NaCl in 25mM TRIS-HCl (pH 8.0)
- Elution profile monitored by A^{280nm} (0.1 AUFS)
- ΔWB of individual fractions.

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(ii) Gel/WB Analysis:

Aliquots of various fractions were taken for NuPAGE gel separation & WB analysis. Due to a fraction collector mishap, I lost a good portion of the column fractions between #1 → ~17. There was enough to analyze by gels. Also, in the case of the pH 8.0 ppt the material (& existing protein by difference) was resuspended in 0.45 ml dH_2O.

Lane	Sample-gel 1	Sample-gel 2
1	CmBVES-Hu63p2L-TM1	8
2	dialyzed CM's (pH 8)	9
3	ppt from dialysis	10
4	Vo I	11
5	Vo II	12
6	4	13
7	5	14
8	6	17
9	7	19
10	STDX	STD1

16.25 µl sample + 6.25 µl 4X LB + 2.5 µl DTT → 70°C, 10'

Following electrophoresis (200V, ~45'), the gel was electroblotted to PDVF (135V, 90') & immunoreactive material visualized & using UP-191-TB #4 as usual (1/1000 dil UP191-TB-#4 / 1/2500 GxR-(AP))

(iii) Low pH Treatment of Fraction 11

The protein content of #10, #11 & #12 was determined using the BioRad Assay. 8 µl of each fraction was run on a 6% NuPAGE gel & visualized by silver staining.

#10	0.22 µg/µl	× ~60 µl ⇒ ~13 µg Total	(220 µg)	~940 µg
#11	0.29 µg/µl	× ~50 µl ⇒ ~14 µg "	(290 µg)	
#12	0.43 µg/µl	× ~60 µl ⇒ 26 µg "	(430 µg)	

16 µl of #11 was mixed w/ 1.6 µl 1.0 M NaOAc (4.5) and incubated o/N @ 4°C. This material was then run in duplicate on a 10% NuPAGE gel as usual (reducing) & ½ of the gel stained by silver & ½ transferred & stained w/ Abs.

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Results:

I previously showed a shift in the Mr of immunoreactive Δsp2LΔTM derived from the Conditioned medium of BVES infection upon acid fractionation to pH 4.5. Since this material appeared unstable (immunoreactive band ↓ w/ time) I reasoned that it might be better to partially purify the pro-form & activate near the end. For this reason, an aliquot of the BVES CM from Δsp2LΔTM was exchanged w/ 25 mM HEPES-KOH (pH 8.0), chromatographed on MonoQ & the elution profile monitored by A^{280} absorbance & WB analysis.

Dialysis of the CM caused minor ppt of protein (~10%) & the clarified Super was fractionated by anion exchange chromatography. A considerable amount of material did not bind to the column & there was no detectable immunoreactive material in the V_0. Gradient elution with a steep NaCl gradient (0->1.0M, 30') resolved multiple A^{280} peaks that eluted between 0->0.5M NaCl. WB analysis of these fractions revealed a strong concentration of immunoreactive material of the expected Mr in fractions 10/11 >> 10/12, well separated from the bulk of the A^{280} absorbing impurities. (Note that a shallower gradient might improve the resolution). This immunoreactive material corresponded w/ an A^{280nm} peak eluting @ ~0.3M NaCl.

Gel analysis of the immunoreactive fractions & silver staining revealed a relatively simple pattern of polypeptides & it was clear from comparison with the immunoblot of the same fractions that which band corresponded (intensity & position (Mr)). In an attempt to reproduce the observation of "activation" in the acid/cold of the CM, fraction #11 (an aliquot) was incubated @ pH 4.5 o/n @ 4°C & the products visualized by both silver stain & WB. The silver stained gel showed a smear, rather than a discrete band, in both #11 & #11, pH 4.5 & a number of additional changes. Alternatively, the Western blot showed a discrete reduction in the observed Mr of the pH 4.5 treated sample, consistent w/ removal of the NH_2-terminal prosegment.

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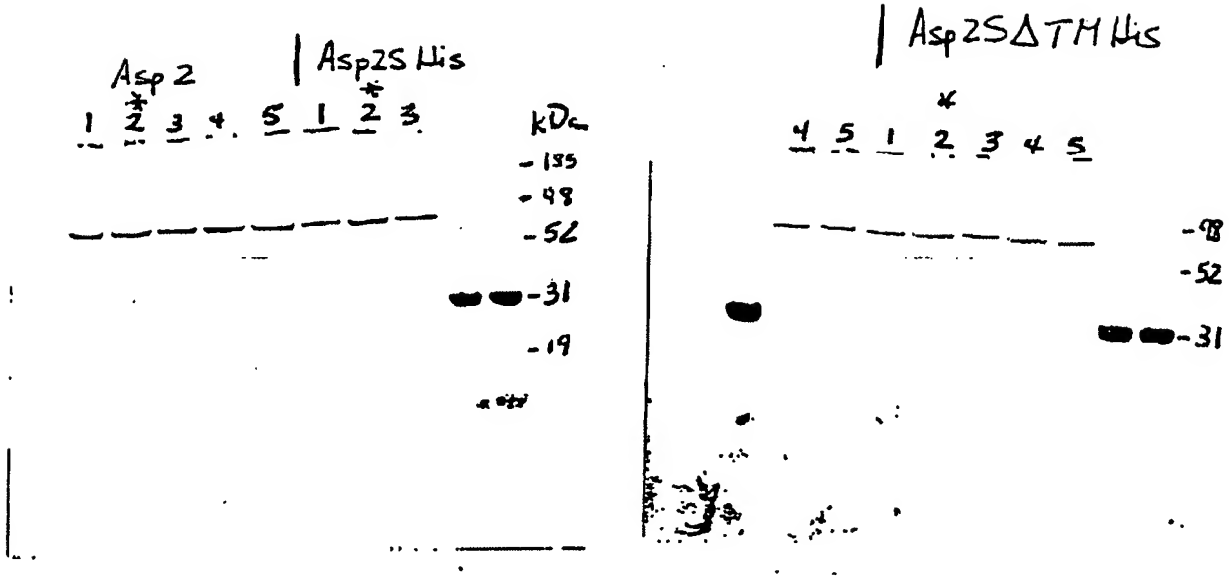
AP Western blots of Baculovirus clones
Probed UP-191 (Asp2) TB#4@1:1000



#2's → Scale up

Exhibit J

TF High Tivrs w/ Asp2ΔTM & Asp2ΔTMHis / p12

These constructs were made by cutting pVH1393 ΔTM & ΔTMHis w/ BamH Not (See p. 87-90) & inserting into p12/V5-His not using vector V5 or His

High fivrs (HS) have been in culture in SF High Tivr. media + Gentamycin for 6 passages & are behaving nicely

Dislodge cells into media, pipet vigorously & count.
Seed ~2 x 10^6 cells / 100mm dish

- Plate 1 dish for each of 3 transient time points (24, 48 hr, 5 day) and 2 for Stables // Construct plus lipisome only
- Rock gently for ~3 mins let cells attach for ~20 min
- Prepare TF reagent: 1 ml SF media
  5 µl 10 µg DNA ATM & ΔTMHis } for each
  20 µl Insectin plus } 60 mm plate
  Vortex 10 sec, Set at r.t ~ 15 mins.
- Remove media from plates — Add DNA/liposome dropwise. Rock @ r.t (29 min) for 4½ hrs
- Add 2 ml SF media → Inc w/ wet paper towels in Sealed bag

- Harvest 24 hr time points — pipet cells into media to loosen. Spin 1.5K 5 mins to pellet the cells. Harvest the Culture media & cells Separately. Store @ -20°C

- Harvest 48 hr time points as above
- Add Zeocin selection to Stables: remove media from 2 x 60mm dishes for each Construct. Resuspend in 10 ml SF media no Antibiotic. Transfer to 150 mm dishes. Allow the cells to sit down ~30 min r.t. Remove media & replace w/ SF media + 400 µg/ml Zeocin

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-Harvest 4½ day (108 hr) time points
Run a 4-12% NuPAGE w/ ~26µl media (from 3 times/2 constructs)
10µl 4X NuPAGE SB
4µl reductant
Stain w/ colloidal blue → no bands in media only control (the conditioned media from all samples show a ladder of bands — one of the darkest bands in the samples & one that seems to intensify with time is running @ ~52 kDa — Western is in order

-Refeed Selection plates — mass killing evident

Run sample gel exactly as above. Electroblot 1 hr. Probe PVDF membrane w/ UP191 (Asp2) @ 1:1000. Develop via AP.
The 52 kDa band is positive!!! Silver stain of the transferred gel shows uneven transfer ∴ The His tagged versions seem to be expressing okay will test 108 hrs is the best of the 3 time points.

-Refeed Zeocin selection plates
Refeed Zeocin " → pockets of adherant cells evident
HJB refed once while I was gone — There are thousands of sparsely placed single cells & attached cell over the plates — no foci evident. (Val Ruff OU7252 is doing a stable transfection in parallel & sees the same thing — even on her liposome (no DNA) plates)
∴ I think we may not have achieved complete Killing of resistant cells ∴ Split 1:2 → 400µg/ml Zeo
→ 600µg/ml Zeo
Floating cells in both 400 & 600 cultures refed w/ respective media
On advice from Invitrogen tech. rep. remove selection & allow foci to form.

Add 25ml media w/o antibiotic to selection dishes
Toss dishes

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Large Scale Transient High five Transfection

Plate 5 x 100mm dishes for each construct: p1Z Asp2ΔTM
p1Z Asp2ΔTMHIS

Cells: 6×10^6/dish
media: 3ml SF media + gentamycin
DNA: 30μg (ΔTM & ΔTM HIS)
Insect in Plus: 60μl

Plate cells, rock 3 min. let cells attach for ~20 mins
Combine media + DNA + liposomes vortex. Incu @ r.t 15 min
Add drop wise to plates. Rock 2 rpms 4 hrs

Add 1ml SF media. Store @ r.t on wet paper towels. MJB to harvest @ 4½ days.

Mike & Monica report tons of protein is being expressed & secreted into the media.

2nd Large Scale Transient

(~4×10^7 cells/confluent T150)

Scale up to 150mm dishes X20
cells: 1.2×10^7
media: 12ml SF media + gentamycin (6ml for transfection)
DNA: 60μg (Asp2ΔTM HIS)
liposomes: 120μl Insectin Plus

Still dividing & happy

⇒ Put 25μg/ml Zeo on one of the 150mm dishes (to select Stables)
Refeed w/ 250μg/ml Zeo
Harvest ~250ml transient conditioned media → Monica for purification

Monica reports β-secretase substrate activity –

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